MOLECULAR IMMUNOLOGY OF BOVINE ISOLATES OF *PASTEURELLA MULTOCIDA* TYPE A

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DECLARATION

I hereby declare that this thesis has been composed by myself and that the work has been carried out by myself except where collaboration is gladly acknowledged.

T.G. Wijewardana
October 1989

DEDICATION

I dedicate this thesis to my husband Ranjith and to our children Sajeewan and Mahesha.

ABSTRACT

Pasteurella multocida type A organisms of bovine origin obtained from the pleural fluid of experimentally infected lambs were found to express two unique outer membrane proteins of molecular masses of 84 and 87 kDa. These proteins were also expressed in bacteria grown *in vitro* under iron restriction in nutrient broth (NB) containing 2,2'-dipyridyl, but not in bacteria grown in NB. Mice were immunized with heat killed organisms which had been grown in NB, NB containing 2,2'-dipyridyl, horse serum and *in vivo*, and good protection was demonstrated. There was a suggestion that *in vitro* grown *P. multocida* expressing these iron regulated proteins (IRP) induced a slightly higher level of protection against challenge but this was not significant on statistical analysis. A greater antibody response was observed in mice immunized with bacteria expressing IRP compared to the other growth conditions as determined by ELISA. However, no antibody response against the 84 and 87 kDa proteins could be demonstrated in Western blots.

Mice vaccinated with NB grown heat killed *P. multocida* induced 100% protection against a homologous challenge of 10 LD_{50}. Vaccination of mice induced antibodies responsible for complement-mediated bactericidal activity of serum and higher ELISA titres to whole cells, compared to the unvaccinated controls. This suggested that the protection was clearly associated with the bactericidal antibodies. The correlation of bactericidal capacity with whole-cell ELISA titres indicated that cell surface antigens were involved in stimulating bactericidal antibodies.

Monoclonal antibodies (mAb) against *P. multocida* type A were produced after vaccination of Balb/c mice with heat killed organisms of NB containing 2,2'-dipyridyl grown cells. An IgM mAb against the capsule agglutinated a capsular extract antigen-coated sheep red blood cells but failed to protect mice. In contrast, an anti-lipopolysaccharide (LPS) mAb of IgG_3 sub class demonstrated complement-mediated bactericidal capacity, protected mice passively against a challenge of 10 LD_{50}, but was not haemagglutinating. This mAb reacted with only 58% of *P. multocida* isolates in ELISA and was shown by an ELISA inhibition test with fractions of LPS to be specific for lipid A of the LPS molecule. The bactericidal capacity of the lipid A reactive mAb against *P. multocida* isolates correlated with the ELISA titres. A clear association between the whole-cell ELISA titres, bactericidal capacity and Western blotting of LPS of different isolates was established.

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CONTENTS

CHAPTER 1

General Introduction

History of the Disease

Pasteurellosis of cattle was first observed by Bollinger in 1878, while in 1880 the causative organism of fowl cholera was isolated by Pasteur. Kitt in 1885 named the organism *Bacterium bipolare multocidum* (Breed *et al* 1957). Having observed the close relationship of the organisms which cause bovine pasteurellosis, fowl cholera and rabbit septicaemia, Huppe in 1886 proposed the name *Bacillus septicaemia* for these bacteria (Buchanan and Murray 1916). Kruse in 1886 introduced the name *Bacillus bovisepticum* (Bain *et al* 1982). Lignierisi in 1901, introduced the generic name of *Pasteurella* for the whole group of organisms which cause pasteurellosis in animals in honour of Pasteur as suggested by Trevisan in 1887 (Bain *et al* 1982). Although Wilson and Miles (1975) preferred *P. septica*, the name *Pasteurella multocida* suggested by Rosenbusch and Merchant (1939) is now established.

P. multocida has been isolated from a wide variety of mammals and birds. It is the cause of haemorrhagic septicaemia of cattle and buffaloes in Southern Europe, North, Central and East Africa, the Near East, and Southern and Southeast Asia (Bain *et al* 1982). It is also associated with pneumonia of cattle, sheep and pigs; fowl cholera and certain forms of mastitis in cattle and sheep (Hussaini 1975). In pneumonic lungs of lambs, however, *P. haemolytica* is more commonly isolated than *P. multocida* (Hamdy *et al* 1959). The importance of *P. multocida* in the aetiology of

pasteurellosis in rabbits reared for both commercial and laboratory purposes is well recognized (Lu *et al* 1978, Percy *et al* 1984; Manning 1984). The most common human infections associated with *Pasteurella* species are those involving the respiratory tract and those resulting from dog and cat bites (Meyer 1958; Carter 1967). In 1977, Johnson and Rumans reported an unusual case of acute epiglottitis in a man and it has also been reported to be a commensal resident of the upper respiratory flora of healthy man (Johnson and Rumans 1977) and animals (Gilmour 1978).

Morphological, cultural and Biochemical Characteristics

Morphological Characteristics

P. multocida is a Gram-negative, non-motile, rod or coccobacillus, which shows a characteristic bipolar staining with Leishman and methylene blue stains. It is aerobic and non-spore-forming, producing fine translucent colonies about one millimeter in diameter with a characteristic musty odour on nutrient agar (Carter 1979). These colonies are non-haemolytic, and do not grow on MacConkey agar (Breed *et al* 1957).

Cultural Characteristics / Colonial Variation

The existence of a large variety of terms to describe similar colonial variations associated with *P. multocida* led Carter (1957) to recommend that the scheme devised by Braun for the colonial variations displayed by other bacteria be adopted for *P. multocida*. Three principle variants were designated and they were mucoid, smooth, and rough. The smooth variants were further differentiated

on the presence or absence of iridescence in oblique light, as smooth iridescent or smooth non-iridescent.

While prevailing types of *P. multocida* isolated from rabbits were mucoid (Webster and Burn 1926) smooth, smooth iridescent and smooth non- iridescent colonial variants have been isolated from fowl cholera (Hughes 1930). The relationship of colonial variation to virulence varies with the species of laboratory animal used. Cultures initiated from mucoid and smooth variants were highly virulent for mice whereas mucoid cultures that tend to autolyse were weakly or moderately virulent (Carter and Bigland 1953). As iridescence was lost, mouse virulence may (Yaw *et al* 1956) or may not (Carter 1957) drop. Low virulence in mice was observed with rough variants of *P. multocida* (Anderson *et al* 1929). Study of colonial dissociation revealed that the mucoid variant was derived from the smooth form (Elberg and Cheng-Lee Ho 1950 ; Carter and Bigland 1953), with the sequence of dissociation being smooth --> mucoid ---> rough (Carter 1957). A similar sequence to this has been observed *in vitro* in a fowl cholera isolate (Heddleston *et al* 1964). Animal passage resulted in the replacement of smooth non-iridescent variants by smooth iridescent culture (Carter 1967; Heddleston *et al* 1964).

Biochemical Characteristics

P. multocida organisms are positive for oxidase (Carter 1984) catalase (Cowan 1974), and indole (Breed *et al* 1957) and produce nitrites from nitrates (Namioka 1978). They are also indole positive (Breed *et al* 1957). They ferment sugars such as glucose,

sucrose, sorbitol and mannitol with acid, but with no gas production. Lactose, maltose and salicin are not fermented, whilst variable fermentation is obtained with arabinose, trehalose and xylose (Cowan 1974). Gelatin is not liquefied (Breed *et al* 1957). In triple sugar iron agar, although acid is produced, no gas or hydrogen sulphide are produced (Carter 1984). Table 1.1 describes the main criteria for the differentiation of *P. multocida* from other closely related species.

Serological Characteristics

Early Attempts at Classification of *P.multocida*

Lignieresi (1901) suggested a "zoological classification" of the species *Pasteurella multocida*, based entirely on the history of isolation of the organism. The existence of more than one serological group in this species was later demonstrated by several workers employing different serological procedures (Cornelius 1929; Yusef 1935; Rosenbusch and Merchant 1939; Little and Lyon 1943; Robert 1947) but none of these classifications were followed up. These are summarized in Table 1.2.

Classification of *P. multocida* by the Capsular Antigen

Isolates of *P. multocida* were later grouped into four different types A, B, C and D on the serological specificity of the capsular substance (Carter 1955; Namioka and Murata 1961a). Carter (1961) reported the existence of a new type, "E", and eliminated the earlier type C (Carter 1963) as it did not represent a single important type or a group (Table 1.2).

Table 1.1 Some basic criteria for the identification of *P. multocida* and closely related species.

Species	Growth on MacConkey	Haemolysis	Indole	Urease
P.multocida	-	-	+	-(a)
P.haemolytica	+	+	-	-
P.haemolytica var *urea*	-	+(b)	-	+
P.pneumotropica	-	-	+	+
P.gallinarum	-	-	-	-

(a)= some exceptions reported

(b)= not as haemolytic as *P.haemolytica*

Classification of *P. multocida* by the Somatic Antigen

The classifications described below are based on the lipopolysaccharides (LPS) of *P. multocida*. Since LPS represent the major surface antigens of Gram-negative bacteria and most LPS are active as endotoxins, the terms O-antigens, endotoxin and LPS are used synonymously (Brade *et al* 1988).

The somatic (O) antigen (the residue left after hydrochloric acid treatment of the cells) was the basis of this classification (Namioka and Murata 1961a; 1961b; 1961c). Within the four capsular types of *P. multocida*, eleven somatic types were identified (Namioka and Murata 1961a; 1961b; 1961c; Namioka and Bruner 1963; Namioka and Murata 1964). The method involved a complex procedure of absorptions for producing type-specific sera, and, therefore, failed to gain wide application (Table 1.2).

Another somatic serotyping system was later introduced by Heddleston *et al* (1972) for fowl cholera isolates. The antigen used was a lipopolysaccharide-protein complex similar but not identical to endotoxin. To date sixteen serotypes have been described using this method (Table 1.2).

Designation of Serotypes

To standardize the method of designating a serotype, Carter and Chengappa (1981) recommended the use of a combination of Carter's (capsular) and Heddleston's (somatic) serological classifications. Thus, for example, a serotype will be designated as A : 3 where A is the capsular antigen and 3 describes the somatic antigen.

Table 1.2: Classification of *P. multocida*

Authors	Procedure	Results
Early Methods		
Cornelius (1929)	Agglutination absorption	Groups I, II, III, IV
Yusef (1935)	Precipitation	Groups I, II, III, IV
Rosenbusch and Merchant (1939)	Agglutination, fermentation of sugars	Groups I, II, III
Little and Lyon (1943)	Slide agglutination	Types 1, 2, 3
Roberts (1947)	Passive protection in mice	Types I, II, III, IV
Serotyping based on capsular substance		
Carter (1955)	Indirect haemagglutination	Types A, B, C, D
Carter (1961)	Indirect haemagglutination	New type E
Carter (1963)	Indirect haemagglutination and passive protection in mice	Non-existence of type C & left with types A, B, D, E
Namioka and Murata (1961a)	Slide agglutination	Types A, B, C, D
Serotyping based on somatic antigens		
Namioka and Murata (1961a, b, c)	Agglutination - adsorption (tube agglutination test)	Somatic types 1, 2, 3, 4, 5, 6
Namioka and Bruner (1963)	Agglutination - adsorption (tube agglutination test)	Somatic types 7, 8, 9, 10
Namioka and Murata (1964)	Agglutination test (tube agglutination test)	Somatic type 11
Heddleston *et al* (1972)	Agar gel diffusion precipitation	Somatic types 1 to 16

Distribution of Serotypes Among Animals

Strains of capsular types A and D are distributed widely with regard to host, while most of the type B and E strains are recovered from cattle, bison and water buffaloes (Carter 1967). An association of somatic type and *P. multocida* infections in different animals was described by Murata and Namioka (1964). According to the earlier classification of somatic : capsular combination, serotype 5:A occurred most commonly as a pathogen of fowl (Namioka and Murata 1961c). Isolates of serotype 9:A were recovered from turkeys and the most prevalent serotype from swine was 1:A. The occurrence of a number of serotypes in several animal species was also reported. Serotype 5:A was recovered from pigs as well as from poultry; and 1:A, which occurred in pigs was also recovered from mice (Namioka and Murata 1963). Isolates belonging to either 6:B or 6:E were associated with cases of haemorrhagic septicaemia in cattle and buffaloes (Carter 1967).

Serotype 3:A (Heddleston : Carter) was found to be the commonest isolate among most animal species in USA (Heddleston *et al* 1972; Blackburn *et al* 1975). Although a similar serotype distribution was observed among isolates of *P. multocida* from avian species in Britain, in addition to serotype 3:A, serotype 1:A also appeared to be prevalent (Curtis 1976). A higher percentage of *P. multocida* isolated from rabbits belonged to type A, while few were type D (Lu *et al* 1978). The prevalence of Heddleston's serotypes 3:A and 12:A has been observed in rabbits in USA and Canada (Brogden 1980 ;Lu *et al* 1983 ; Percy *et al* 1984). In another study 3:A was recovered

most frequently from lungs of swine at slaughter (Pijoan *et al* 1983).

Laboratory Animal Models for the Disease

Pasteurella multocida is pathogenic for a wide range of hosts. In pathogenicity, virulence and immunological studies the laboratory animals most commonly used are mice, rabbits, chickens and turkeys because of their high degree of susceptibility to experimental infection by a variety of *P. multocida* serotypes (Carter 1967).

Mice

Collins (1973) studied the pattern of growth of *P. multocida* in vaccinated and normal mice. The rapid multiplication of the organism (minimum *in vivo* generation time 15 min) extracellularly in the peritoneal cavity explained the high virulence of this organism for the mouse.

However the immune response of mice to *P. multocida* was different from that of chicks. Killed capsulated cells of *P. multocida* induced a better immunity than non-capsulated cells in mice. Immunity was induced by the intraperitoneal route but not by the subcutaneous route. However, a high degree of immunity was induced in chicks with both capsulated and non-capsulated cells of *P. multocida*, regardless of the route of inoculation. The mouse model therefore was considered to be unsuitable for the determination of immunogenic differences among isolates of *P.multocida* (Heddleston and Rebers 1969).

Okerman *et al* (1979) used an 8 hour broth culture containing 10^9 cfu/ml to infect mice with isolates of *P. multocida*. Mice were injected with 0.5 ml of a ten fold dilution of the culture broth, mortality was recorded for 7 days and the median lethal dose (LD_{50}) was calculated. Isolates originating from the septicaemic form of pasteurellosis in rabbits were highly virulent for mice, while those from typical snuffles,a local nasal infection,were less virulent. They also exposed mice to an aerosol of an 8 hour broth culture. Mortalities in mice were recorded for up to 10 days. Highly virulent strains killed all the mice within 3 days, less virulent ones killed 50% of mice within 9 days, while non-virulent strains did not kill any mice (Okerman *et al* 1979).

Mice were used to compare the immunogenicity of different strains of *P. multocida* type A from bovine pneumonia (Abdullahi 1987). Mice were infected with 10 LD_{50} of *P. multocida* i.p following immunization with two doses of vaccine inoculated s.c two weeks apart.

Rabbits

Webster (1928) infected rabbits intranasally with a culture of *P. multocida* (then named as *Bacterium lepisepticum*). During the periods of observation, the longest being 5 months, normal rabbits reacted in different ways to the experimental infection. Some rabbits became either short-term or long-term carriers, some developed snuffles of varying duration; while others developed snuffles followed by pneumonia and signs of general infection after a few days or a few weeks. The authors concluded that the effects

observed depended upon the degree of resistance of the individual animals and the virulence of *P. multocida*.

To determine the most effective route of inoculation of *P. multocida* in order to produce pneumonic lesions in rabbits with serotypes 3:A and 3:D, Percy *et al* (1986) infected rabbits by aerosol exposure, intracerebral and intravenous inoculations. Any rabbits that survived were killed on the 14^{th} day of observation and specimens from all animals were examined microscopically for lesions in the lower respiratory tract. They concluded that the intratracheal route was the best method for producing pneumonic lesions in rabbits.

The pathogenesis of *P. multocida* serotype 3:A in pasteurella-free rabbits has been studied with different doses ranging from 3.4×10^6 to 1.8×10^{10} organisms administered intranasally and subcutaneously (Lu *et al* 1982). The degree of pathogenicity was dose and route dependent.

Although mice or rabbits have been widely used in these experiments, the advantages of use of the natural host from which *P. multocida* was isolated has also been advocated (Heddleston and Rebers 1969; Penn and Nagy 1976).

Antigenic Analysis of *P.multocida*

Gram-negative bacteria have been defined serologically according to the antigenic composition of capsule and cell-wall antigens. Some strains of bacteria possess a capsule composed of polysaccharide, while others a capsular matrix of glycoprotein overlie the bacterial cell membrane. The cell membrane or the cell

envelope of Gram-negative bacteria is comprised of at least three distinct layers, namely, the inner or cytoplasmic membrane, the peptidoglycan layer and associated periplasmic space, and the outer membrane, which acts as a selective barrier on the exterior surface of the cell (Smyth 1985). A complex trilaminar structure of lipoproteins, phospholipids and lipopolysaccharides is found in the outer membrane of Gram-negative bacteria (Figure 1.1).

A number of methods have been developed for the fractionation of Gram-negative bacterial cell envelopes and for the separation of inner and outer membranes, thereby allowing the isolation of outer membrane with or without peptidoglycan (Schnaitman 1971;Osborn and Munson 1974; Lugtenberg and Van Alphen 1983). On the other hand, simple methods such as mild heat treatment, or extraction of bacteria with buffers of differing pH and ionic strengths have been used to remove and prepare the capsular substances of Gram-negative bacteria(Hancock and Poxton 1988). The capsular and cell-wall components isolated from *P. multocida* have been used to classify them into different serological groups and to study their serological and immunological responses in different animal species.

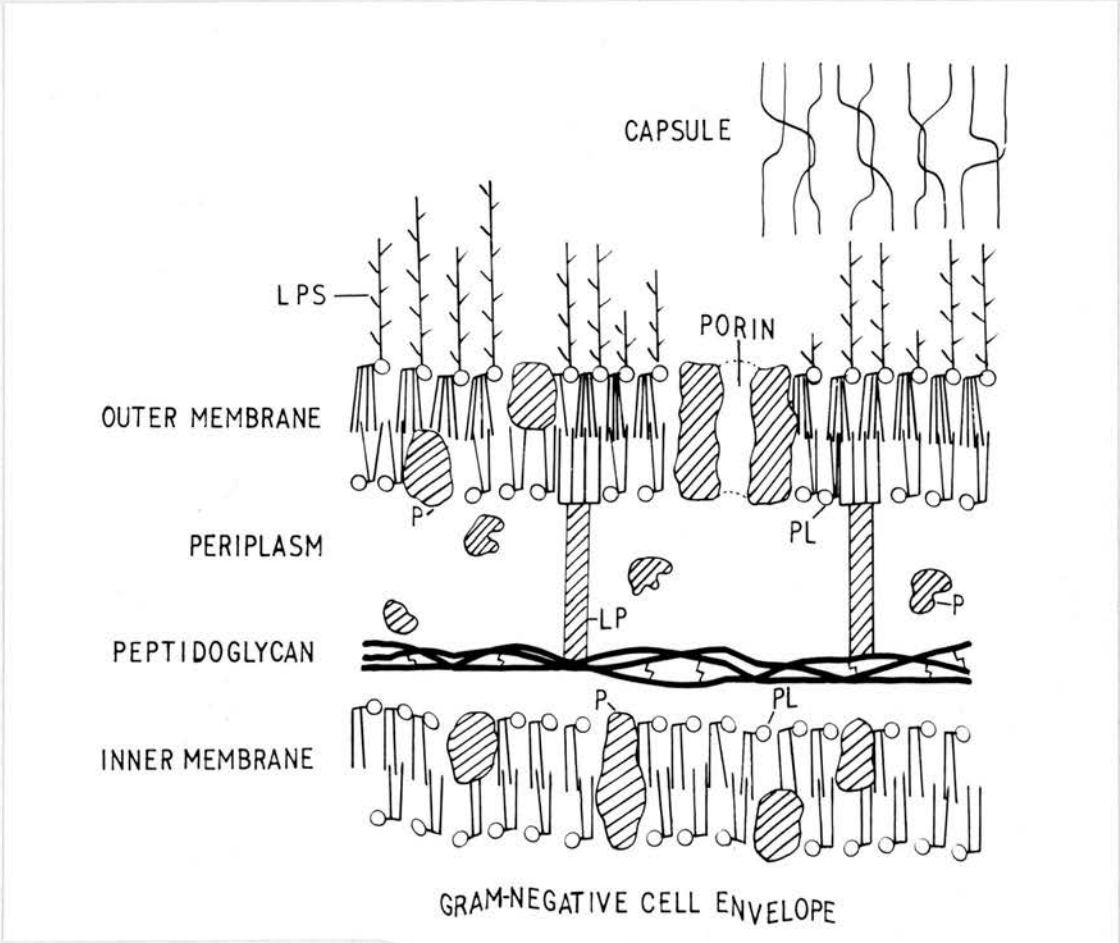
Studies on the Capsular Polysaccharide of *P. multocida*

Extraction of type specific polysaccharide substance from the capsule of *P. multocida* was described by Hoffenriech (1928) and Pirosky (1938). Later, isolates of *P. multocida* were consigned into different serological groups according to their capsular composition (Carter 1952; Carter and Annau 1953; Carter 1955).

Fig. 1.1. The cell envelope of a Gram-negative bacterium (Courtesy of Dr. I.R. Poxton)

LP: lipoprotein; LPS: lipopolysaccharide;
P: protein; PL: phospholipid

Fig. 1.1.


CAPSULE
LPS
PORIN
OUTER MEMBRANE
P
PL
PERIPLASM
LP
P
PEPTIDOGLYCAN
P
PL
INNER MEMBRANE
GRAM-NEGATIVE CELL ENVELOPE

The ability of a crude capsular polysaccharide to induce protection in both chickens and mice was reported (Yaw and Kakavas 1957). Knox and Bain(1960) purified a polysaccharide from a saline extract of *P. multocida* type 1 (Robert's) which contained fructose, mannose, glucose and glucosamine. The crude saline extract contained varying amounts of proteins and polysaccharides. Absorption of rabbit and cattle immune sera with this purified polysaccharide abolished its ability to precipitate antigen in agarose gel and reduced but did not abolish the protection afforded by these sera (Knox and Bain 1960). Thus the capsular antigen was important in producing immunity to *P. multocida*.

Using immunodiffusion and immunoelectrophoresis techniques, 18 soluble antigens were identified in the extracts of haemorrhagic septicaemia(HS) strains of *P. multocida* (Prince and Smith 1966a). Two of these antigens were capsular in origin and were designated as alpha (protein) and beta (polysaccharide) which were believed to correspond to the protein and polysaccharide antigens of Knox and Bain (1960). Comparison of soluble extracts of *P. multocida* (HS strain) with extracts from eight other Gram-negative species in gel diffusion tests demonstrated the existence of five common antigens (Prince and Smith 1966b). The capsular antigens, i.e. alpha and beta described in the previous study, were not shared by any of the other Gram-negative species tested. In another study Prince and Smith (1966c) compared the soluble antigens of *P. multocida* strains belonging to Carter's types A,B and E. Both alpha and beta capsular antigens were type specific in Carter's type B and E strains, but the capsular antigen alpha had some antigenic determinants which

were non-type-specific and were shared with a non-capsular component of other serotypes.

In a comparative study of the saline and phenol water extracts of *P. multocida* type B and E, capsular polysaccharide was recognised as the type specific antigen, while LPS was the antigen common to both serotypes (Penn and Nagy 1974). Both extracts contained capsular antigen and LPS in varying amounts. Saline extract consisted mainly of capsular antigen while phenol water extract contained mostly LPS . It was concluded that the protection afforded by these serotypes was largely type-specific and that the capsular polysaccharide must therefore, be a protective antigen. The importance of the capsular antigen in the protection of cattle has been confirmed by the successful absorption of protective antibodies from serum raised against whole cells of *P. multocida* in cattle by the purified capsular substance of type B and E of *P. multocida* (Nagy and Penn 1974). Purification of capsular antigen was felt to be essential since minor contamination of immunizing preparations with LPS might elicit the production of anti-LPS antibodies (Penn and Nagy 1976).The nature of this antigen, purified by solvent fractionation was studied. The antigen exhibited a molecular weight of approximately two million, was heat stable, was resistant to pronase digestion, and did not absorb light at 280 nm which suggested the antigen was not protein in nature. These properties also suggested the antigen would be a good candidate for inclusion in a vaccine production.

Studies on Lipopolysaccharide (LPS) of *P.multocida*

Isolation and characterization of LPS of *P.multocida*

A Boivin type antigen (endotoxin) was recovered from smooth and rough variants of an avian strain of *P. multocida* by Pirosky (1938). The Boivin antigen was toxic, protective, and serologically specific. The occurrence of type specific lipopolysaccharides containing aldoheptose sugars in *P. septica (P. multocida)* was later reported by Maclennan and Rondle (1957). This LPS was toxic in mice, heat stable and serologically specific. Phenol water-extracted LPS from type 1 (Robert's) *P. multocida* yielded galactose, glucose, glucosamine and a heptose sugar (Bain and Knox 1961). An Australian strain of *P. multocida*, which by conventional mouse protection and haemagglutination tests was identified as type 1, yielded an LPS with different properties. The capsular substances adsorbed to erythrocytes for the identification of capsular types were later recognised as LPS (Bain and Knox 1961; Carter and Rappay 1963).

LPS isolated from non-capsulated avirulent mutants of two fowl cholera strains of *P. multocida* were immunogenic and toxic in chickens (Heddleston *et al* 1966). Intravenous injection of 0.16 mg amounts of LPS produced clinical signs in chickens similar to those observed in acute fowl cholera. Similar antigens were also isolated from virulent, capsulated, haemorrhagic septicaemic strains (Rebers *et al* 1967). A comparative study of the free endotoxin and Westphal-type LPS of capsulated and non-capsulated strains of *P. multocida* revealed that the free endotoxins readily induced antibody formation and active immunity, but the LPS did

not (Rebers and Heddleston 1974). The free endotoxin for this study was obtained by washing agar-grown cells with cold formalinized saline.

Structure of LPS

Enterobacterial LPS consist of three regions which can be distinguished chemically, biologically and genetically. These three regions are the O-specific side chain, core oligosaccharide and lipid A (Figure 1.2). Mutants with a genetic defect in the biosynthesis of complete LPS, and containing incomplete LPS which lacks the O-chains, or O-chains and part of the core have been described (Galanos and Luderitz 1984 and Brade *et al* 1988).

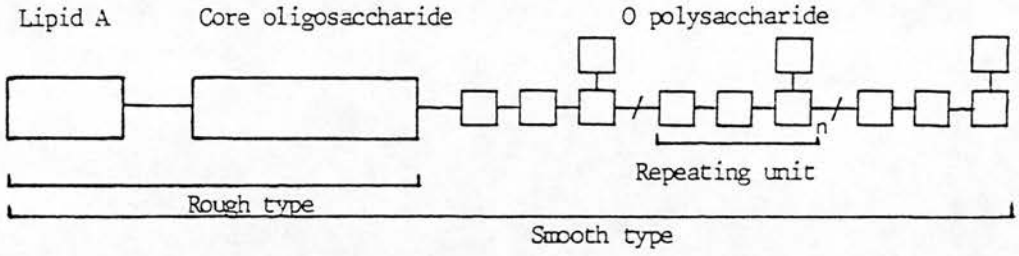


Fig. 1.2. Diagrammatic representation of lipopolysaccharide (Poxton and Arbuthnott 1990).

Chemical and physical analysis of LPS of 13 serotypes of *P. multocida* (Heddleston's) showed that all LPS contained glucose, 2-keto-3 deoxyoctonate and heptose, D-glycero-D-mannoheptose and L-glycero-D-mannoheptose (Rimler *et al* 1984).

Characterization of LPS from five isolates of *P. multocida* from rabbits by SDS-PAGE (silver stained), Western blotting and ELISA revealed that the LPS of these isolates contained two types of antigens, a non-specific and a sero-specific antigen. The LPS of

four isolates each had a different sero-specific antigen. The non-sero-specific antigen was expressed in two isolates and was the only demonstrable LPS antigen in one other isolate. The LPS of these isolates resembled those of semi-rough strains of enterobacteria (Manning *et al* 1986).

Immunogenicity of LPS

Loss of immunogenicity of whole cells and free-endotoxin on treatment with 50% phenol indicated the requirement for the induction of immunity of a protein component in each. An LPS-protein complex was, therefore, postulated to be present in the endotoxin, the immunologic specificity being determined by the carbohydrate components of the LPS and the protein being required for induction of immunity (Rebers and Heddleston 1974; Heddleston and Rebers 1975). Fractionation of a culture supernate of *P. multocida* with ether resulted in a "glycoprotein-like" preparation while phenol provided a "LPS-like" preparation. The fraction extracted by ether was an excellent immunogen in mice and phenol extraction destroyed the immunogenicity of the supernate (Srivastava and Foster 1977). An immunogenic fraction of a saline extract of *P. multocida* consisted chiefly of a high molecular weight protein-carbohydrate complex (Ganfield *et al* 1976). Isolation of an immunogenic complex of protein-LPS from a saline extract of *P. multocida* in turkeys was also reported by Syuto and Matsumoto (1982).

Comparison of the Heddleston's heat stable antigen (1972) and the Westphal type LPS of *P. multocida* in an agar-gel diffusion

precipitin test indicated that the major component of the heat stable antigen responsible for the type specificity was LPS (Brogden and Rebers 1978). An LPS preparation that was free of capsular antigen as confirmed by an agar-gel diffusion precipitin test was not immunogenic in mice and rabbits when compared to formalinized bacteria (Schmerr and Rebers 1979). Similarly both purified and crude LPS were able to induce protection in chickens but not in mice and rabbits (Rebers *et al* 1980). Purified LPS in this study was obtained by digestion of crude LPS with deoxyribonuclease and ribonuclease enzymes followed by buoyant density gradient centrifugation in caesium chloride. Rebers *et al* (1980) observed that serum produced against the purified LPS in chickens had a stronger serologic reaction and was more effective in passive protection experiments in chickens.

LPS of serotypes 2 and 5 were found to be antigenically similar when examined by the passive haemagglutination test and also antisera against purified serotype 2 LPS passively immunized 6 of 10 poults against the serotype 5 strain. The LPS of serotype 5 of fowl cholera and serotype 2 of haemorrhagic septicaemia strains of *P. multocida* have similar chemical structures (Rimler *et al* 1984). Similar cross-reactions between LPS of serotypes 4 and 12 of *P. multocida* of rabbit origin in an ELISA indicated that the LPS of these two serotypes were the common shared antigen (Cary *et al* 1984).

Studies on Protein or Protein Complexes of *P. multocida*

Bain (1955) employed potassium thiocyanate (KSCN) to extract *P. multocida* type 1 (Robert's). Fractionation of the KSCN extract gave two components; a protective protein-polysaccharide complex and a polysaccharide that behaved as a hapten. A crude KSCN extract from *P. multocida* type 1 (Robert's) consisting of a protein-carbohydrate complex was immunogenic and non-toxic to mice given in 1 mg amounts i.p, while a sodium chloride extract consisting of a protein- carbohydrate complex was toxic to mice at doses as low as 42 ug i.p (Mukkur and Nilakantan 1972). In another study , KSCN extract of *P. multocida* serotype 3 (Heddleston) of avian origin was found to be immunogenic in chickens against the homologous as well as against one heterologous strain (Gaunt *et al* 1977).

Immunization of calves with KSCN extract of *P. multocida* type A resulted in production of antibodies which were agglutinating, haemagglutinating and bactericidal to both *P. multocida* and *P. haemolytica* (Mukkur 1977). Treatment of this extract with proteolytic enzymes like papain, trypsin, pronase and proteinase k, or heating at 56°C to 121°C destroyed its immunogenicity, suggesting that the important immunogenic determinants in the extracts were protein in nature (Mukkur and Pyliotis 1981). Different fractions obtained by gel filtration and ultracentrifugation of KSCN extract expressed varying levels of immunogenic activity in mice, which were significantly less than that of the crude extract. These were therefore a multiplicity of protective antigens associated with *P. multocida* and the synergistic interaction between them (Mukkur *et al* 1982).

A ribosomal fraction, obtained after disruption of *P. multocida* cells in a french pressure cell, protected chickens and mice (Baba 1977). Further studies on ribosomal fractions indicated the presence of a ribosomal-LPS mixture, in which ribosomes served as immunomodulators or as adjuvant while LPS induced protection in chickens (Phillip *et al* 1981; Phillip and Rimler 1984).

Examination of cytoplasmic and cell envelope associated antigens of *P. multocida* serotype 1 (Heddleston's) by crossed immunoelectrophoresis demonstrated at least 55 cytoplasmic and 19 cell envelope associated antigens (Bhasin and Lapointe-Shaw 1980). This would indicate that humoral and/or cell-mediated immune responses elicited by such crude preparations as culture filtrate, cell wall, cytoplasm and other complex antigen preparations extracted from whole cells of *P. multocida* were probably composite reactions to more than one antigen. They also reported the capacity of the formalin-killed whole cells of *P. multocida* to elicit an immune response to internally situated soluble antigens. This indicated that *P. multocida* cells were relatively fragile and disintegrated readily *in vivo* to expose internal antigenic components.

SDS-PAGE Analysis of *P. multocida* Antigens

Lugtenberg *et al* (1984) suggested the use of the SDS-PAGE pattern of LPS and cell surface proteins instead of the guinea-pig skin test to predict pathogenicity of strains of *P. multocida* isolated from atrophic rhinitis in swine. Characterization of the cell envelope protein patterns of 34 isolates of *P. multocida* from

swine revealed three distinct patterns and the electrophoretic mobility of a major protein "H" in the range of an approximate molecular weight of 36kDa was used to differentiate them into groups according to their cell envelope protein pattern (Lugtenberg *et al* 1984; Lugtenberg *et al* 1986). Recognition of the same protein in a doublet by an immunoblot with sera raised against whole cells of *P. multocida* in pigs indicated that they were among the major surface-exposed proteins. Following electrophoretic separation of *P. multocida* cell envelope, the gels were reacted with immune sera from pigs in a gel-radioimmunoassay. An antigen which appeared as a smear with a relatively low electrophoretic mobility was considered to be an immunogenic complex consisting of protein 'H' and LPS (Lugtenberg *et al* 1986).

In contra-distinction to the studies with the swine isolates of *P. multocida* the outer-membrane protein pattern of bovine *P. multocida* type A did not differentiate the isolates into serological groups or correlate with their pathogenicity in mice. Also there were no differences in the outer-membrane patterns among the typical *P. multocida* type A, atypical *P. multocida* type A and taxon 13 strains (Abdullahi 1987). On the other hand, outer-membrane protein profiles of isolates of *P. multocida* belonging to serotypes 1, 3 and 12 (Heddleston's) on SDS-PAGE differed from one another and these differences may have accounted for the lack of heterologous protection among isolates of *in vitro* grown *P. multocida* (Corbett *et al* 1983; 1984).

Sera raised against a KSCN extract of *P. multocida* serotype 3:A in rabbits were used to identify the outer-membrane immunogens by

radioimmunoprecipitation and Western blotting (Lu *et al* 1988a). Of the 18 proteins recognised by these sera, the major antibody response was directed against a 27,000 molecular weight (27kDa) outer-membrane protein together with 37.5, 49.5, 58.7 and 64.4kDa outer-membrane proteins. Absorption of the immune sera with the intact *P. multocida* organisms resulted in a significant reduction of antibody activity directed against these proteins, especially the 37.5kDa. Elution of an antibody against the 37.5kDa immunogen from the surface of the *P. multocida* organisms indicated this immunogen was exposed on the cell surface and was accessible to antibody (Lu *et al* 1988a). A monoclonal antibody to this 37.5kDa protein recognised 36 of 153 rabbit clinical isolates and was distributed among the major somatic types 3, 10, 12, and 15 and also capsular types A and D, suggesting the possible use of this outer-membrane protein as a vaccine (Lu *et al* 1988b).

Vaccines

Pasteur in 1880 produced an attenuated vaccine from live cultures from cases of fowl cholera by prolonged growth on artificial media. These protected fowls from subsequent infection. This method, however, was not reproducible. Since that time the search for new vaccines to control pasteurellosis has continued.

Bacterins/Chicken Embryo Vaccine

Phenol-killed broth-grown bacterins (Hilbert and Tax 1938) and chicken embryo vaccines (Carter 1950) have been used to control fowl cholera in ducks. Immunogenicity of the chicken embryo

vaccine was found to be superior to the Hilbert and Tax's phenol-killed broth bacterin and other commercial bacterins as it protected the largest percentage of ducklings against infection with virulent *P. multocida* cultures (Dougherty 1953).

Adjuvant Vaccines

With the introduction of adjuvants a considerable improvement in the efficacy of the bacterins has been observed (Freund and Bonanto 1944). The value of light mineral oil or water-in-oil emulsion vaccines for enhancing and prolonging antibody response to bacteria had been stressed by Freund *et al* in 1948. This was supported by the findings of Bain (1954) when cattle immunized with conventional formalin-killed bacterin succumbed to infection while those immunized with bacterin in oil-adjuvant were immune.

Later, the oil adjuvanted vaccines were subjected to detailed studies by many research workers (Vancheswara *et al* 1955; Bain 1959; Dhanda and Lall 1958; Dhanda et al 1960; Mall and Nilakanthan 1971). Because of its efficacy, safety and stability, the oil adjuvant vaccine was recommended for the control of haemorrhagic septicaemia (Bain *et al* 1982).

An oil adjuvant vaccine containing killed whole cells of *P. multocida* induced better immunity in chickens than either an aqueous-suspended, alum-precipitated, chicken embryo, or a broth bacterin or an adjuvanted vaccine of capsular substance (Heddleston and Hall 1958). Heddleston and Reisinger (1960) showed that by increasing the concentration of bacterin and aluminium hydroxide gel in the aluminium hydroxide adsorbed, killed fowl cholera

vaccine, a much longer duration of immunity of at least 52 weeks could be attained.

Bacterins prepared from *P. multocida* type A killed by formalin and B-propiolactone conferred protection in birds which lasted five months after vaccination, regardless of the differences in the killing agent (Chute *et al* 1962). In contrast, a heat-inactivated bacterin containing type A *P. multocida* strain induced better immunity in turkeys than formalinized bacterins irrespective of the kind of adjuvant used(Bhasin and Biberstein 1968).

Induction of Cross-Protection

P. multocida shows a marked antigenic diversity and the host environment apparently influences the development of protective antigens (Heddleston and Rebers 1972;1974). Use of host grown *P. multocida* to study the immune response in chickens and turkeys has therefore been considered as important. Bacterins prepared from *P. multocida* grown in the tissues of birds demonstrated homologous as well as heterologous protection whereas a bacterin prepared with bacteria grown on dextrose starch agar failed to induce heterologous protection revealing that *P. multocida* produce a wider spectrum of antigens *in vivo* rather than *in vitro* (Heddleston and Rebers 1972). The cross-immunity induced in turkeys was host-specific, as the antigen responsible was produced in turkeys, only when *P. multocida* was grown in embryonating turkey eggs and not when it was grown in embryonating chicken eggs or in mouse tissues (Heddleston and Rebers 1974). The cross-protection afforded by a bacterin prepared with *P. multocida* originating from

infected turkey or chicken tissue was lost when *P. multocida* in the bacterin were replaced by repeatedly subcultured cells (Rebers and Heddleston 1977). Crude liver homogenates as well as infected blood from turkeys which died of experimental fowl cholera produced by serotypes 1 or 3 of *P. multocida* induced cross-protection. In contrast to this, neither the washed bacterial cells nor bacteria-free plasma from infected turkeys induced significant cross-protection (Rimler *et al* 1979). Most of the cross-protection factors were bound to the bacterial cell and could be easily removed by simple washing.

Rimler *et al* (1979) searched for appropriate conditions under which *P. multocida* could be grown *in vitro* that might have had the equivalent antigenic content to induce protection and cross-protection as live attenuated vaccines. It was found that B-vitamins enhanced this ability while certain inorganic salts (e.g $(NH_4)_2SO_4$, KH_2PO_4, $NaHCO_3$, $MnSO_4$,$FeSO_4$) repressed it. Plasma of normal turkeys contained a compound that was responsible for expression and maintenance of this heterologous protection. Under *in vitro* conditions growth temperature also influenced expression of cross-protecting antigens which was enhanced at 41.5°C and repressed at 37°C. If grown and maintained under appropriate conditions, *in vitro* grown *P. multocida* can be as immunogenic as tissue and live attenuated vaccines. Rimler *et al* (1979) successfully maintained the cross protection factors of avian *P. multocida in vitro* through at least nine serial passages by growing the organisms in media containing 50% turkey blood. Separation of *P. multocida* organisms from infected turkey blood has

been carried out satisfactorily by density gradient centrifugation in sucrose. Complete lysis of host grown *P. multocida* by freeze-thawing and treatment with lytic solutions (ie.DNAse, hyaluronidase, EDTA or Triton X-100) have not influenced the ability of the lysate to induce homologous as well as heterologous protection (Rimler and Rhoades 1981).

The level of protection (either homologous or heterologous) induced by a vaccine varied with the quality of the particular immunogens that have been incorporated in the vaccine (Rimler and Rhoades 1981). Differential centrifugation and density gradient centrifugation were employed to separate the cross-protection factors from the complete lysate of *P. multocida* (Brogden and Rimler 1982a). Homologous and heterologous immunizing activities were found both in the soluble and in the pelleted portions of the lysate. The immunogenic activity of the cross-protection factors was greatly reduced after trypsin treatment and was completely abolished after pepsin treatment, but was not affected by heating at 56°C for 1 hour. This suggested that the antigen was protein in nature (Brogden and Rimler 1982a). Examination of the pellet obtained after centrifugation of the complete lysate by electron microcopy revealed it consisted of small membrane vesicles, ranging in size from 0.05 um to 1.0 um. These vesicles also had a characteristic trilaminar membranous appearance, similar to those reported from other Gram-negative bacteria treated similarly (Brogden and Rimler 1982b). An attempt has been made to release protein from the membrane vesicles that may contain cross-protection factors. Vaccines made from combining solubilized

membrane vesicles with complete lysate supernatant fluid produced various degrees of protection against heterologous infection in turkeys (Brogden and Rimler 1983). Vaccines containing membrane vesicle material solubilized by KSCN or sodium lauroyl sarcosinate, when combined with complete lysate supernate, protected turkeys as did complete lysate. The authors concluded that the cross-protection factor of turkey-grown *P. multocida* was expected to be an antigen that turkeys would respond to during a natural infection.

Vaccination of Mice with *P. multocida*

Of the vaccines which contained formalin-killed, heat-killed whole cells, LPS and capsular substance of bovine *P. multocida* type A in adjuvant, only formalin and heat killed whole cell vaccine induced protection in mice (Abdullahi 1987). However, the antigens responsible for this protection could not be identified by the techniques such as Enzyme-Linked Immunosorbent Assay (ELISA), immuno-blotting and fused rocket-immunoelectrophoresis.

Vaccines prepared from the tissues of mice dying of pasteurellosis induced by four different capsular serotypes (A, B, D and E) of *P. multocida* provided complete homologous protection with all serotypes and complete cross-protection between the haemorrhagic septicaemia serotypes B and E. Although this complete heterologous protection between serotypes B and E correlated with somatic antigen specificity, the homologous protection observed in other serotypes demonstrated involvement of other antigens (i.e capsular or complex protein) besides serotype specific ones (Rimler and Boycott 1979).

The possible existence of a common immunizing antigen between *P. multocida* type A and D had been revealed by active immunization and passive protection experiments (Cameron *et al* 1978; 1980). Vaccines containing more than four strains of *P.multocida* failed to induce protection even against the vaccinating strain (Cameron and Bester 1983). However, hyperimmune sheep antiserum to a type D strain protected mice against infection with a number of type A strains. Later, an alum-precipitated vaccine was formulated incorporating this type D strain and a selected strain of type A (Cameron and Bester 1984). The vaccine induced production of antibodies in sheep that conferred homologous protection in mice as well as affording heterologous protection against a variety of type A and D strains and a number of untypable strains of *P. multocida*. These studies suggest that *P. multocida* express an antigen that could be common to more than one serotype.

Live Attenuated Vaccines

Any killing of organisms for purposes of vaccine production may result in loss of important immunogens (Chengappa *et al* 1980). A live vaccine may reduce or eliminate this loss of protective immunogens. A live *P. multocida* vaccine administered in drinking water induced immunity against heterologous infection in turkeys (Maheswaran *et al* 1973; Heddleston *et al* 1975; and Coates *et al* 1977). A wild type *P. multocida* type A isolated from a rabbit suffering from respiratory disease was used to produce a streptomycin-dependent mutant and the rabbits were vaccinated with either 4.5×10^8 or 5.0×10^8 organisms/ml subcutaneously or

intranasally (Chengappa *et al* 1980). This vaccine elicited complete protection against homologous infection with virulent wild type *P. multocida* . A similar vaccine incorporating live streptomycin-dependent *P. multocida* 3:A (Heddleston's) and live streptomycin-dependent *P. haemolytica* A1 induced better protection than the commercial bacterin when tested against a virulent homologous infection in calves (Catt *et al* 1985). In another study immunization of rabbits with a streptomycin-dependent strain of *P. multocida* type 12:A (Heddleston) elicited detectable antibody production to somatic antigens of both 12:A and heterologous strain 3:A (Percy *et al* 1985).

P. multocida (B:3,4) isolated from a fallow deer in England which was less virulent for calves was used as a live vaccine to protect calves against experimental haemorrhagic septicaemia (B:2) infection (Myint *et al* 1987).

Subcellular Components of *P.multocida* as Vaccine

Although the use of bacterins and live attenuated vaccines are generally effective against *P. multocida* infection, the disease still occurs in vaccinated animals. Therefore, attempts have been made to identify subcellular components of *P. multocida* that would induce protection, in the hope of developing vaccines of higher potency than those currently in use.

Vaccines Containing Capsular Polysaccharides

A heat-extracted crude capsular polysaccharide has been used to protected mice against heterologous infection (Carter and Annau

1953) and chickens and mice against a homologous infection of *P. multocida* (Yaw and Kakavas 1957). Dhanda (1958) reported that of the five different fractions (carbohydrate-protein, protein, toxin, LPS and protein-free capsular carbohydrate) obtained after treatment with different solvents, the protein-free capsular carbohydrate and the LPS fractions were not immunogenic in mice, chickens and rabbits. However, Bain (1958) found both LPS and protein-free capsular polysaccharide fractions were to be immunogenic in mice and cattle.

A vaccine of emulsified cell-free capsular extract of *P. multocida* induced effective immunity against a virulent infection of *P. multocida* in chickens for at least 25 weeks (Heddleston and Hall 1958). A capsular antigen purified from a crude saline extract of type B and E *P. multocida* provided active and passive protection in mice (Penn and Nagy 1974; Nagy and Penn 1974). Adsorption of anti-whole cell bovine serum by the purified capsular antigen, eliminated its protective ability in mice against a homologous infection. The capsular antigen was therefore considered to be the protective antigen in cattle. Although the capsular antigen was protective in cattle in aluminium-hydroxide gel adjuvant, it was poorly immunogenic in rabbits (Penn and Nagy 1976).

The immunogenicity of capsular polysaccharide was also investigated by Kodama *et al* (1981). Although the crude capsular antigen extracted from *P. multocida* provided protection in turkeys against homologous infection, the purified polysaccharide antigen obtained by treating the crude capsular antigen with

cetylpyridinium chloride was non-immunogenic. The crude capsular antigen was a polysaccharide-protein complex of which the immunogenicity was lost by acid hydrolysis but not affected by treatment with chloroform, trypsin or heat. The apparent stability of this crude capsular antigen was considered as an advantage in vaccine production (Kodama *et al* 1981).

A protein-carbohydrate antigen purified from the crude capsular substance by chromatography induced the formation of antibodies in rabbits and turkeys (Syuto and Matsumoto 1982). The same antigens isolated from serotypes 1 and 3 (Heddleston) *P. multocida* type A, although possessing similar physico-chemical properties, were found to be immunogenically distinct from each other as analysed by the agarose gel diffusion precipitation test and cross-protection experiments in turkeys (Kajikawa and Matsumoto 1984).

Vaccines Containing LPS or LPS/Protein Complexes

An antigen which resembled endotoxins, extracted with cold formalinized saline from smooth and rough variants of *P. multocida* protected birds against fowl cholera infection(Heddleston *et al* 1966). Using the same method an LPS-protein complex was isolated from a virulent, encapsulated strain of *P. multocida*, which in 15 ug amounts protected mice against a homologous infection (Rebers *et al* 1967). Treatment of the LPS with phenol destroyed the protein moiety of the LPS-protein complex rendering it non-immunogenic (Heddleston and Rebers 1975 and Schmerr and Rebers 1979).

A KSCN extract of *P. multocida* serotype 3 (Heddleston's) protected chickens against homologous as well as one heterologous

strain (serotype 1). Emulsification of the extract in Freund's incomplete adjuvant (FIA) increased its efficacy. A component in the KSCN extract of the two serotypes were found to be identical in the agar-gel diffusion precipitation test (Gaunt *et al* 1977). Cross-protection between *P. multocida* type A and *P. haemolytica* serotype A1 in mice immunized with KSCN extract of *P. multocida* suggest a sharing of common immunogens between the two species (Mukkur 1977).

In calves too, this KSCN extract produced higher antibody responses when it was inoculated with FIA than those inoculated with Tris-saline buffer (Mukkur 1978). A protein-LPS complex isolated from KSCN extract of *P. multocida* was found to be more immunogenic than the crude extract in mice (Ryu and Kaeberle 1986).

Studies carried out in rabbits with the KSCN extract of *P. multocida* serotype 3:A provided significant protection against homologous experimental infection, and this vaccine appeared to be safe when inoculated by the intranasal or the intramuscular routes (Lu *et al* 1987a).

A vaccine containing a ribosomal fraction of *P. multocida* serotype 8:A (Heddleston) protected mice and chickens against virulent, homologous infection (Baba 1977). He confirmed that pure LPS is not an immunogen in mice and suggested that the protective power of LPS-protein complex observed in previous studies (Heddleston *et al* 1966, Rebers and Heddleston 1974) could be due to the presence of ribosomes as contaminants in the preparations. The requirement of LPS in ribosomal vaccines for the development of protection was confirmed by Phillip and Rimler (1984). The

addition of a serotype 1 (Heddleston) LPS to LPS-free affinity purified ribosomes obtained from *P. multocida* resulted in a vaccine that protected against a homologous infection but replacement of 1:A LPS with heterologous serotype 5:A LPS in LPS-free affinity purified ribosomes did not protect chickens against a virulent serotype 1 infection, but produced antibodies to serotype 5. This indicated that protection was conferred by specific LPS. The protective ability of the ribosomal vaccine was conserved when ribosomes were substituted by other fungal or bacterial (*Aspergillus fumigatus*, *Brucella abortus*) ribosomes but not when replaced by chicken liver ribosomes, revealing that ribosomes or their components have to be foreign to the host to potentiate LPS immunogenicity (Phillip and Rimler 1984).

Growth of *P.multocida* under Iron Restriction Conditions

One common and essential factor in all infections is the ability of the invading pathogen to multiply successfully in host tissues. This ability is known to be greatly influenced by the availability of iron. Most of the iron in the body of mammals is found intracellularly as ferritin, haemosiderin or haem and extracellularly in serum attached to high-affinity iron-binding proteins, transferrin in blood and lymph and lactoferrin in external secretions and milk. A related protein called ovotransferrin occurs in avian egg white (Griffiths 1985). The amount of free iron available to bacteria therefore is extremely small. Even with this restriction, those bacteria which multiply successfully and establish an infection must be able to develop

mechanisms for assimilating protein-bound iron or for acquiring it from liberated haem. Most micro-organisms have been found to produce specific high-affinity iron-binding compounds termed siderophores, which chelate ferric ions into an assimilable form (Barclay 1985). When the bacteria have to adapt to a severely iron-restricted environment by producing siderophores they also produce a number of high-molecular mass outer-membrane proteins. In *E. coli* these proteins are usually designated according to their relative molecular masses 83, 81, 78 and 74kDa proteins.

Iron regulated outer-membrane proteins were demonstrated when *E.coli* O111 was grown in broth containing ovotransferrin or *in vivo* during infection (Overbeeke and Lugtenberg 1980). Other pathogenic bacteria have also been shown to produce extra outer-membrane proteins when grown under iron-restricted conditions *in vitro* (Griffiths 1985). An outer-membrane protein of *Pseudomonas aeruginosa* described as ferripyochelin-binding protein (FBP) was involved in iron acquisition when complexed with the *Pseudomonas* siderophore, pyochelin. It was detected as a major outer-membrane protein in iron-starved, glucose grown cells (Sokol and Woods 1983). *Proteus mirabilis* and *Klebsiella pneumoniae* isolated directly from the urine of patients with urinary tract infection expressed extra high molecular mass outer-membrane proteins. These proteins corresponded to proteins that were induced in the iron-depleted medium and disappeared when the organisms were grown in iron-rich medium *in vitro* (Shand *et al* 1985). Recognition of these proteins by the patient's own serum revealed that these proteins were highly antigenic.

A fish pathogen, *Vibrio anguillarum*, also produced an outer-membrane protein (86kDa) under iron restricted conditions (Actis *et al* 1985). The role of outer-membrane proteins in the immune mechanisms were studied in certain instances. Anti-FBP antibodies were found to be opsonic and protected mice against *P.aeruginosa* infection (Sokol and Wood 1985). Passive immunization of turkeys with antibodies to iron-regulated outer-membrane proteins of *E.coli* protected them from experimental colisepticaemia (Bolin and Jensen 1987).

Recent studies on *P. multocida* indicated that iron deprivation induced four new outer-membrane proteins of approximate molecular weight 33, 60 65 and 71kDa and decreased the presence of outer-membrane proteins 48k and 58kDa as compared to iron replete cells. Active immunization of mice with these outer-membrane preparations provided homologous protection but not heterologous protection (Corbett *et al* 1985). They also reported that no phenolate or hydroxymate-type siderophores could be detected in the supernates of iron-starved cells. Secretion of a siderophore into the culture medium by *P. multocida* type A strains under conditions of iron deprivation was first reported by Hu *et al* (1986). This siderophore with a novel chemical structure was given a trivial name "multocidin". As *P. multocida* is known to be a very virulent organism to many animal species, apart from producing siderophores, it must produce outer-membrane proteins to assimilate iron from bound proteins of the host. These proteins if incorporated into vaccines may provide a higher level of immunity in animals against homologous as well as heterologous infection.

Evidence suggested that *P. multocida* grown *in vivo* in turkeys produced better immunogens which induced homologous and heterologous protection than *in vitro* grown cells which conferred homologous protection only (Heddleston and Rebers 1972; 1974; Rebers and Heddleston 1977 and Rimler *et al* 1979).

Immunity

Effector mechanism(s) of Immunity to *P.multocida* Infection

Unvaccinated laboratory animals (rabbits, mice and turkeys) are highly sensitive to many strains of *P. multocida* (Carter 1967). The natural hosts (cattle and birds) seem to be incapable of mounting any resistance to the progressing infection, even when very small numbers of virulent organisms are introduced into the tissues. This results in a highly lethal infection involving the lungs, liver, spleen and blood (Collin 1973). Attempts to increase host resistance by active immunization is practiced as a routine. As most avian or bovine strains of *P. multocida* are also virulent for mice, this host has been used for the experimental assay of *P. multocida* vaccines (Murata *et al* 1964; Heddleston and Rebers 1969; Rimler and Boycott 1979). In such protection tests the development of acquired resistance can be inferred from an 80% or better survival of the vaccinated mice in the face of a more than 80% mortality in the unvaccinated controls. The mechanism involved in the expression of this acquired resistance seems to be humoral in nature, since passive protection can readily be achieved by inoculating hyperimmune sera (Carter 1967; Collins 1977; Nathanson *et al* 1980 and Lu *et al* 1987a). However, the exact mode of action

of the opsonic, antitoxic or bactericidal antibodies in overcoming infection is still not clear (Collins 1973; Woolcock and Collins 1976).

Phagocytosis

In *P. multocida* infection, phagocytosis of pathogenic organisms by cells of non-immune, susceptible animals is slight. However, there is evidence for opsonic action by specific antibodies both *in vivo* and *in vitro* correlating with enhanced protection against experimental infection (Carter 1964; 1967 and Collins 1973). On the other hand, in passive protection experiments, inclusion of antibodies within the peritoneal cavity did not always result in the immediate inactivation of the bacterial population, but it limited the free spread of the organisms from the initial site of infection into the blood (Bain *et al* 1982; Woolcock and Collins 1976). Also it was evident that the immune process did not directly involve the mononuclear cellular defences but only polymorphonuclear leukocytes (Woolcock and Collins 1976; Collins and Woolcock 1976). Failure of specific antibody and complement to kill or even inhibit the growth of *P. multocida* and the apparent inability of specific antibody to enhance granulocyte phagocytosis *in vitro* indicated that antibody may not have a significant role in controlling invasive infections in rabbits (Hoffing *et al* 1979). Resistance of *P. multocida* to granulocyte phagocytosis was found only in some virulent strains.

Rush *et al* (1981) reported that the virulence factors of *P. multocida* type A, which promoted resistance to rabbit

polymorphonuclear leukocyte phagocytosis and killing, were not associated with the hyaluronic acid capsule. In another study the resistance of *P. multocida* type A to rabbit polymorphonuclear leucocyte was attributed to the presence of hyaluronic acid capsule (Maheswaran and Thies 1979; Anderson *et al* 1984). In contrast to these findings, three isolates of *P. multocida* with substantial differences in virulence (LD_{50}) for mice were all inactivated by normal mouse peritoneal macrophages provided that the organisms were opsonized with specific hyperimmune serum (Collins *et al* 1983). Ryu *et al* (1984) reported that the inhibitory factor present in type A *P. multocida* capsule which determined its effect on polymorphonuclear leukocytes was not hyaluronic acid but was a heat stable, saline extractable capsular material of molecular mass greater than 300kDa. In another study, the morphology of *P. multocida* was attributed to the differences observed in the association of bacteria with bovine alveolar macrophages and killing of bacteria (Ashfaq and Campbell 1986). In this study specific antibody and/or complement enhanced the association of the rough strain of *P. multocida* with macrophages and also increased the killing effect of macrophages on the bacteria, while in a smooth strain of *P. multocida* only association of bacteria with macrophages was observed. The role of specific antibodies in the phagocytosis of *P. multocida* is not clear as there are many reports of non-immune adherence of pasteurellae to polymorphonuclear leukocytes or macrophages and intracellular killing of *P. multocida* (Rush *et al* 1981; Anderson *et al* 1984 and Ashfaq and Campbell

1986). Very little is known about the importance of complement in both the opsonization of *P.multocida* and in the stimulation of intracellular killing.

Bactericidal Activity of Serum

Bactericidal antibodies to *P. multocida* have not been conclusively demonstrated (Carter 1967). Although it is accepted that immunity to *P. multocida* is humoral in nature, which mechanism(s) operate however, remains unanswered. Exposure of many strains of Gram-negative bacteria (i.e, *E. coli, Salmonella*) to serum results in loss of viability and even in destruction of the bacterial cell. The role of complement and its activation via the classical or the alternative pathways by Gram-negative bacteria is well known. The activation of complement via the classical pathway requires recognition of bacterial surface antigens by certain antibody classes whereas activation of the alternative pathway can be initiated in the absence of antigen-antibody interactions, by certain structural characteristics of the cell surface (Taylor 1983). In a complement-dependent bactericidal assay the relative importance of IgM and IgG as bactericidal antibodies were emphasized (Schulkind *et al* 1972). In the sera of mice that were successfully immunized with a KSCN extract of *P. multocida* against an experimental infection, bactericidal activity was found to be localized in the IgM and IgG fractions (Mukkur 1979). Clinical isolates of *P. haemolytica* A1 were shown to be susceptible to bactericidal activity of antibodies in adult cattle sera when these antibodies were combined with complement (MacDonald *et al* 1983).

Presence of bactericidal antibodies in the sera and lung washings of lambs that recovered from *P. haemolytica* A2 infection was demonstrated by Sutherland (1988). The presence of these antibodies correlated with the resistance of lambs to *P.haemolytica* infection. On the other hand, resistance of clinical isolates of both *P.haemolytica* and *P.multocida* to serum was also reported which could be attributed to the virulence of these organisms (Blau *et al* 1987). Although there are indications as to the susceptibility of *P. multocida* to serum killing which operates via the classical pathway (Griffiths 1974; Mukkur 1979 and Blau *et al* 1987) very little is known on this aspect of mechanism(s) of immunity to *P. multocida* .

Measurement of humoral immunity to *P.multocida*

A number of procedures have been developed for the measurement of humoral immunity to *P. multocida*. These are: the agglutination, haemagglutination, serum bactericidal and mouse passive protection tests (Carter 1967). Carter (1964) found a correlation between haemagglutination titre to *P. multocida* and the passive protection capacity of anti-pasteurella serum in mice. Protection tests in mice and the indirect haemagglutination test to measure antibodies to type E and B *P. multocida* in cattle and buffaloes have been extensively used in many countries (Perreau *et al* 1964; Bain *et al* 1982; De Alwis *et al* 1986; Wijewardana *et al* 1986). Failure to determine the immune status of chickens and turkeys to *P. multocida* by the passive protection tests in mice, the agar-double diffusion test or serum plate-agglutination test was reported by Heddleston

and Watko (1965). The antibody response of the vaccinated birds detected by the haemagglutination test had no bearing on the immune status of the birds (Bhasin and Biberstein 1968). These two studies reflected that protective antibodies in birds were not detected by the usual serological methods. The indirect haemagglutination test was the best indicator of the immune status of non-vaccinated sheep, while the agar agglutination test was found to be the best for measuring the immune status of vaccinated sheep (Dua and Panduranga 1978).

It is evident from this review of the literature, that the antigens involved in protection against *P. multocida* infection have not been well defined. As a result currently- available vaccines give varying degrees of protection and the disease still remains as a problem in many species of animals. The experiments described in this thesis were therefore aimed at identifying antigens which would give a better immunity, and the possible effector mechanisms of immunity in *P. multocida* in mice. The objectives were therefore:

a) to study the outer membrane proteins of *P. multocida* type A of bovine origin grown in *in vivo* and *in vitro* under iron restricted conditions.

b) to evaluate the immune response of mice to cells grown in *in vivo* and *in vitro* under iron restricted conditons.

c) to study the effector mechanisms of immunity to *P. multocida* in mice.

d) to produce and characterize monoclonal antibodies against type A *P. multocida* with a view to identifying the protective cell-surface antigens involved in *P. multocida*.

CHAPTER 2

General Materials and Methods

2.1 Media for Isolation and Cultivation of *P. multocida*

2.1.1 Blood Agar with 5% Sheep Blood

Dehydrated blood agar base 40g (Gibco) was suspended in 1 litre of distilled water and sterilized by autoclaving at 121°C for 15 min. Sterile sheep blood 50ml, collected in 4% citrate, was added after cooling the medium to between 45°C and 50°C and the mixture dispensed in 20ml volumes aseptically into sterile petri dishes.

2.1.2 Dextrose Starch Agar (DSA)

Dehydrated DSA 65g (Gibco) was suspended in 1 litre of distilled water and sterilized by autoclaving at 121°C for 15 min and 25ml volumes poured into sterile petri dishes.

2.1.3 Nutrient Broth (NB)

This was prepared by dissolving 25g of NB dehydrated powder (Oxoid) in 1 litre of distilled water and dispensing in the required volumes either into bottles or conical flasks and sterilized by autoclaving at 121°C for 15 min.

2.1.4 Brain Heart Infusion Broth (BHIB)

Dehydrated BHIB powder 37g (Oxoid) was dissolved in 1 litre of distilled water; dispensed in the required volumes into bottles and sterilized by autoclaving at 121°C for 15 min.

2.2 Isolates of *P. multocida*

The isolates of *P. multocida* used in this study were obtained from veterinary investigation centres. Some strains from the culture collection of Abdullahi (1987) identified and characterized biochemically and serologically as *P. multocida* type A were also used. All these strains were isolated from cattle with a history of pneumonia or upper respiratory tract infection.

2.2.1 Identification of Isolates of *P.multocida*

a) Biochemical tests using API 20E System

Isolates were identified as *P. multocida* based on their biochemical characteristics using the API 20E system (API Laboratory Products Ltd., Grafton Way, Basingstoke, Hants.). A bacterial suspension prepared by emulsifying 3-4 isolated colonies of *P. multocida* from DSA or BA in 1% horse serum in distilled water was used to inoculate the tubes of the API 20E strips. The biochemical tests were carried out according to the manufacturer's instructions and the results were interpreted using the API 20E index.

b) Staphylococcal hyaluronidase decapsulation test

In order to determine the capsular type of *P. multocida* isolates the method by Carter and Rundell (1975) was followed. Briefly, a colony of *P. multocida* from a DSA or BA plate was streaked transversely across the whole plate of a freshly prepared BA plate. A hyaluronidase producing strain of *Staphylococcus aureus* was then streaked at right angles to the

pasteurella streaks. The plate was placed in a moist container and incubated at 37°C for 24 h. The hyaluronidase effect manifested as a reduction in the size of the Pasteurella colonies in the region adjacent to the staphylococcal streak, indicating that those colonies of *P. multocida* belonged to capsular type A.

2.2.2 Storage of Isolates of *P.multocida*

All the isolates were stored lyophilized and/or frozen at -70°C. To store at -70°C, the cultures were grown in NB, NB with 50% heat-inactivated filter-sterilized horse serum (HS) at 37°C for 6 h or in sheep blood overnight (o/n). Volumes (1ml) were dispensed into plastic freezing vials (NUNC, Denmark) and stored at -70°C.

2.3 Preparation of *P. multocida* antigens

2.3.1 Heat killed Organisms (HKO)

An iridescent colony from a DSA plate was inoculated into 10ml of NB and incubated at 37°C for 6 h on a multishaker. The 10ml broth culture was inoculated into 1 litre of NB and incubated at 37°C for 18 h with continuous shaking. The cells were harvested by centrifugation at 5000g for 20 min, washed once in phosphate buffered saline (PBS: 0.137M NaCl, 0.003M KCl, 0.001M KH_2PO_4 and 0.08M Na_2HPO_4, pH 7.4) and resuspended in 10ml of PBS. The cells were checked for purity by plating onto a blood agar plate and then killed by heating at 60°C for 90 min. After heat treatment the cells were checked for viability by streaking onto a BA plate and [illegible] then lyophilized.

2.3.2 Crude Capsular Antigen (CCA)

Preparation of crude capsular antigen was similar to that described under the IHA test. The mucoid culture was washed off a DSA plate with 3ml 0.01M PBS, pH 6.0 and 1.0ml of PBS pH 6.0, containing 15 National Formulary Units of testicular hyaluronidase (Sigma Chemical Co.) was added. The suspension was placed in a water bath at 37°C for 3-4 h. It was then heated at 56°C for 30 min, after which the bacteria were removed by centrifugation at 5,000g for 20 min. The supernatant consisting of CCA was stored at -20°C in 0.5ml aliquots.

2.3.3 Lipopolysaccharide (LPS)

a) Extraction of LPS by aqueous phenol

Lipopolysaccharides were extracted from dried cells of *P. multocida* using the method originally described by Westphal *et al* (1952) with certain modifications. NB (50ml) was inoculated with a loopful of *P. multocida* (W674) from a BA plate and incubated at 37°C for 6 h with shaking. This 50ml broth culture was subsequently inoculated into 5 litres of NB and incubated at 37°C overnight with shaking. The cells were harvested by centrifugation at 14000g for 20 min, washed once in distilled water and resuspended in 50ml of distilled water. The cells were checked for purity and then lyophilized.

Dried bacterial mass was resuspended to a concentration of 5% w/v in distilled water and heated to 68°C in a water bath. An equal volume of 90% w/v aqueous phenol was also heated to 68°C and added to the prewarmed bacterial suspension mixed and

stirred at 68°C for 15 min. The mixture was transferred into centrifuge tubes and cooled on ice for 10 min, to allow phase separation to occur. The tubes were centrifuged at 8000g for 20 min, to complete phase separation. The upper aqueous phase, containing the LPS, was carefully removed with a pasteur pipette and the remaining suspension was retreated at 68°C with the original volume of distilled water. Both upper aqueous phases collected, were pooled and dialysed against running tap water for 48 h to remove phenol. The dialysed liquid was centrifuged at 10,000g for 20 min to remove the insoluble deposit before being concentrated to 50% of its original volume with a rotary evaporator. The concentrated solution was centrifuged at 100,000g for 90 min, when LPS sedimented to form a clear gelatinous pellet. The pellet was resuspended in distilled water with the aid of a syringe fitted with a 23 gauge needle and recentrifuged as above. The final pellet was resuspended in 1ml of distilled water and lyophilized.

b) Preparation of LPS by Proteinase K enzyme digestion

Preparation of LPS in amounts suitable for analysis by PAGE was essentially according to the method described by Hitchcock and Brown (1983). *P. multocida* was grown in 10ml NB at 37°C overnight and the cells were washed once and resuspended in PBS to an absorbance at 525nm of between 0.5 and 0.6. Cells from 1.5ml of bacterial suspension were sedimented in a microcentrifuge at 1000g for 3mins. The pellet was resuspended in 50 µl of SDS-PAGE sample buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue in

0.062M Tris-HCl buffer pH 6.8) and heated to 100°C for 10 min. After cooling Proteinase K (25µg, Protease type XI, Sigma) in 10µl of sample buffer was added, and this mixture incubated at 60°C for 60 min. The resulting preparations were analysed by PAGE omitting SDS from both stacking and separating gels.

2.3.4 Preparation of Outer Membranes (OM) by Sarkosyl Extraction

The OM were prepared according to the method described by Shand *et al* (1985). A colony of *P. multocida* from a DSA plate was inoculated into 30ml of NB and incubated at 37°C for 6 h with shaking. Cells harvested from 30ml, were washed once in PBS, resuspended in PBS and inoculated into 3 litres of either NB, BHIB or any other specialized medium described in the relevant chapter. An 18 h culture grown at 37°C with agitation was harvested by centrifugation at 14,000g for 20 min, washed twice in PBS and resuspended in 30ml distilled water. The cells were disrupted by sonication for 3 bursts of 60s using 9mm probe (MSE 150W Ultrasonic Disintegrator). The sonicated cell suspension was centrifuged at 5,000g for 20 min, to sediment the unbroken cells. The supernatant was collected and centrifuged at 40,000g to pellet the cell envelopes. The envelope pellet was washed twice and resuspended in 0.1M Tris buffer, pH 7.4, to 8ml and extracted with 2ml of 10% Sarkosyl v/v in distilled water (Sodium n-lauroyl sarcosinate 30% W/V solution, Sigma Chemical Co.) for 30 min at 37°C. The insoluble

OM was pelleted by centrifugation at 100,000g for 60 min, washed once in 10ml Tris buffer (0.01M pH 7.4), resuspended in 1ml of Tris buffer and stored at -20°C.

2.4 Inoculation of mice with *P. multocida*

2.4.1 Animals

Swiss white mice bred at MRI were used in these experiments, unless otherwise stated. The mice were of either sex and 4-6 weeks old at the commencement of the experiments.

2.4.2 Determination of LD_{50} of *P.multocida* Isolates in Mice

Colonies of *P. multocida* from BA or DSA plates were inoculated into 10ml of NB and incubated at 37°C for 6 h with shaking. A series of ten-fold dilutions 10^{-1} to 10^{-10} of the NB culture were made in peptone water and a viable count was performed by the method of Miles and Misra (1938). Volumes (1ml) of each dilution were injected intraperitoneally into groups of 5 mice. The number of deaths per group was recorded up to 48 h post-infection. The LD_{50} value was calculated by the method of Karber (1939).

2.4.3 Vaccination and Challenge of Mice

a) Vaccines

Vaccines were prepared containing 5mg/ml of HKO. Lyophilized cells were suspended to 10mg/ml in distilled water to which was added an equal volume adjuvant (90% Bayol F (Esso), 10% Arlacel (Sigma)) and the mixture homogenized until a stable emulsion was formed.

b) Vaccination of Mice

Mice were inoculated with 0.2ml of vaccine subcutaneously (s.c.) under the loose skin of the groin on day 0. A booster dose of 0.2ml of vaccine was given on day 14.

c) Challenge of Mice

Mice were challenged i.p with 0.1ml of a live 6 h broth culture of *P. multocida* on day 28. The challenge dose consisted of either 10 LD_{50} or a series of LD_{50} of *P. multocida*. An equal number of unvaccinated (control) mice were also inoculated with the same challenge dose in every experiment.

Blood was collected from the tail vein of each mouse before being challenged. Blood was allowed to clot at room temperature overnight. Serum was separated and stored at -20°C.

The number of deaths in each group of mice was recorded up to 48 h after challenge with live *P. multocida*. Surviving mice were killed by placing them in a carbon dioxide chamber. Blood was collected at post mortem from the inferior vena cava of each mouse (using a 1ml syringe and 21 gauge needle). Pooled or individual blood samples were allowed to clot overnight and serum was separated and stored at -20°C.

2.5 Production of rabbit anti-*P. multocida* hyperimmune serum

Rabbits (lopear) of either sex between the ages of 4-6 months, bred at MRI, were used to raise serum against *P. multocida* type A.

P.multocida was seeded heavily onto 2 BA plates in order to obtain a confluent growth and incubated in a moist chamber at

37°C overnight. The growth was harvested with 10ml of saline using a bent sealed pasteur pipette to scrape gently the surface of the agar. The bacteria were harvested by centrifugation and resuspended in 10ml of saline.

Purity and viability tests of the cell suspension were carried out as described previously. A viable count of the cell suspension was performed by the method of Miles and Misra (1938). The cell suspension was then diluted to 50ml with 0.3% formal saline and incubated at 37°C overnight to kill the cells. Following a sterility check a bacterial suspension containing 10^9 colony forming units (cfu)/ml was used to immunize rabbits. Rabbits were bled from the ear vein and the serum tested for the presence of antibodies to *P. multocida* by the IHA test.

Inoculations were carried out according to the schedule in Table 2.1. Seven days after the final inoculation of formalin killed organisms, 1.0ml of a live 6 h broth culture (washed once in saline) was given intravenously (i.v.). A test bleed from the marginal ear vein was carried out 7 days following the live inoculation. Three days later the rabbits were bled by cardiac puncture under terminal anaesthesia and blood allowed to clot at room temperature overnight. Serum was separated and stored at -20°C in 5ml aliquots.

2.6 Techniques

2.6.1 Indirect Haemagglutination Test (IHA)

The test was performed essantially according to the method described by Carter (1955, 1972a) with certain modifications.

Table 2.1. Immunization schedule for the production of hyperimmune serum against *P. multocida* type A in rabbits

Day	Volume of killed cell suspension (10^9 cfu/ml)	Route of inoculation
1	1.0ml	subcutaneous (s.c.)
6	0.5ml	intravenous (i.v.)
10	1.0ml	" (i.v.)
14	1.5ml	" (i.v.)
18	2.0ml	" (i.v.)
22	3.0ml	" (i.v.)
26	5.0ml	" (i.v.)
33	1.0ml	" (i.v.)

Soluble capsular antigen extracted by hyaluronidase was used in this test.

Confluent growth of *P. multocida* from a DSA plate was washed off the plate with 3ml of 0.15M phosphate buffered saline (PBS) at pH 6.0. To the bacterial suspension was added 1ml of PBS pH 6.0, containing 15 National Formulary Units of hyaluronidase and incubated at 37°C for 4 h in a water bath. The bacterial cells were then separated by centrifugation at 4,000g for 30 min. The supernate was transferred to another tube and 0.1ml of packed sheep red blood cells (SRBC) fixed in 1% **glutaraldehyde** (Sawada *et al* 1982) added. The suspension was incubated for a further 2 h in a water bath at 37°C or overnight at 4°C (Carter 1984). The sensitized SRBC were removed by centrifugation at 1,000g for 5 min and washed 3 times with 10ml of saline. The packed cells were then resuspended in 20ml of PBS to give a final concentration of 0.5%. Doubling dilutions of test sera were made in u-bottomed microtitre plates (Sterilin) using 50µl of 0.3% formal saline as the diluent. Equal volumes (50µl) of sensitized SRBC suspension were added to each well and the plate shaken gently and incubated for 1-2 h at room temperature. Sensitized cells were incubated with standard positive and negative serum and unsensitized cells with test serum and diluent as controls.

Coarse, agglutinated SRBC dispersed throughout the bottom of the well, was interpreted as a positive reaction while unagglutinated SRBC settling to the centre giving a

characteristic button appearance was considered as a negative reaction. The IHA titre was expressed as the reciprocal of the highest dilution of serum indicating a positive reaction.

2.6.2 Enzyme-linked Immunosorbent Assay (ELISA)

The method originally described by Engvall and Perlmann (1972) was the basis of this assay. ELISA used in this study was based on the method of Engvall and Perlmann (1972). *P. multocida* whole cells were used as antigen. A confluent growth of *P. multocida* from a DSA plate was removed in 3ml of saline. The cells were harvested by centrifugation at 5,000g for 15 min and washed once in 10ml of saline. These cells were resuspended in 3ml of 0.5% formal saline, and diluted in 0.05M Carbonate/bicarbonate buffer pH 9.6. Wells of microtitre plates (type 129A, Dynatech Laboratories Ltd.) were coated with 100µl of antigen suspension by incubating overnight at 4°C and washed 3 times with PBS/Tween (PBS pH 7.4 containing 0.05% Tween 20). The final wash was left on the plate for 3 min before it was decanted. Samples were diluted in PBS/Tween and 100µl volumes were incubated in antigen coated wells for 3 h at 37°C. The serum samples were decanted and the plate washed 3 times with PBS/Tween. Volumes (100µl) of sheep anti-mouse IgG conjugated with Horseradish peroxidase (Scottish Antibody Production Unit, Lanarkshire, Scotland) (diluted 1 in 200 in PBS/Tween) were added to each well and incubated for 1 h at 37°C. The plates were washed 3 times in PBS/Tween. Finally 100µl of fresh substrate solution was added to each well. This consisted of 4mg of ortho-phenylenediamine (Sigma) and 4µl of H_2O_2 per 10ml

of 0.05M sodium phosphate - 0.024M citric acid buffer pH 5.0. The reaction was terminated by adding 40µl of 2.3M H_2SO_4/well after 5 min. The optical densities of each well were read at 492nm on a Titertek Multiscan (Flow Laboratories, Irvine, Ayrshire).

2.6.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was employed to separate the OMP of *P. multocida* based on their molecular weight and to analyze the LPS of *P. multocida*. The procedure followed here was a modification of the method of Laemmli (1970) described by Poxton and Brown (1979). The details of the techniques were as follows:

2.6.3.1 Composition of buffers/reagents

Separating gel buffer,

0.75M Tris-HCl pH 8.8

0.2% SDS

Stacking gel buffer,

0.25M Tris-HCl pH 6.8

0.2% SDS

Acrylamide stock Solution (40%)

100g acrylamide (elecrophoresis grade)

2.7g methylene bis-acrylamide (elecrophoresis grade)

Electrode buffer, pH 8.3

0.025M Tris

0.192M glycine

0.1% SDS

Sample buffer, (double strength)

0.125M Tris-HCl pH 6.8

4% SDS

20% glycerol

2% 2-mecracaptoethanol

0.002% bromophenol blue

2.6.3.2 Composition of polyacrylamide gel

Acrylamide concentration in the separating gel was either 10% or 14% as indicated in Table 2.2.

2.6.3.3 Procedure

Electrophoresis was performed at room temperature (20^{o}C) on a discontinuous vertical slab gel (170x140mm) prepared with the aid of glass plate sandwich assembly, in an electrophoresis apparatus (Raven Scientific Ltd., Haverhill, Suffolk, U.K.)

A 10% Separating gel was prepared by adding the accelerator APS and the catalyst TEMED (NNN'N' tetramethylethylene diamine) into the deaerated mixture of distilled water, separating gel buffer and acrylamide (Table 2.2). The gel was poured slowly between glass plates using a glass pipette and overlaid with water saturated butan-2-ol and allowed to polymerise for 30 min.

The water-saturated butanol was removed from the surface of the polymerised separating gel and the stacking gel (Table 2.2)

Table 2.2. Composition of Polyacrylamide gels.

Buffer/reagent	Volume (ml) to give acrylamide concentration of: 10%	14%	4% (stacking gel)
Distilled water	6.95	3.45	3.50
Separating gel buffer	17.50	17.50	----
Stacking gel buffer	----	----	5.00
Acrylamide stock solution (40%)	8.75	12.25	1.00
TEMED	0.05	0.05	0.02
Ammonium persulphate (15mg/ml) (APS)	1.75	1.75	0.50

mixture prepared in a similar manner to the separating gel poured on top. A suitable comb was inserted initially at an angle to exclude air bubbles, and the gel allowed to polymerise.

Samples were prepared for electrophoresis by heating equal volumes of sample and double strength sample buffer at 100°C for 3 min. Samples were applied to the wells filled with electrode buffer and electrophoresis was carried out first at 60V until the samples had entered the separating gel, and subsequently at 150V until the dye front had reached 7.5cm from the top of the separating gel.

LPS was analysed on 14% polyacrylamide gels, omitting SDS from both the stacking and separating gel buffers. The gels were stained for proteins with Coomassie brilliant-blue (sequential staining method) and with Silver Stain for LPS by the methods described by Hancock and Poxton (1988).

2.6.4 Western Blotting

Comparison of serological responses of mice to different vaccines of *P. multocida* and characterisation of monoclonal antibodies were carried out by the "Western Blotting" technique, first described by Towbin *et al* (1979). Proteins or LPS separated on PAGE were electrophoretically transferred to nitrocellulose (NC) membrane (Schleicher and Schuell, GMBH, D-3354 Dassel, West Germany) in transfer buffer (0.025M Tris, 0.2M glycine and 25% aqueous methanol, pH 8.3) at 40mA for 16 h in an immunoblot apparatus. In order to ascertain transfer had taken place, NC membrane was stained with 0.2% Ponceau S (Sigma)

in distilled water for 10 min. and molecular mass markers marked on blot, then rinsed in water and cleared. The membrane was washed for 10 min in Tris-buffered saline (TBS) (20mM Tris, 500mM NaCl, pH 7.5) and placed in 3% w/v gelatin in TBS for 45 min to block free binding sites. After blocking with gelatin, NC membrane was probed with primary antibody (polyclonal serum, ascitic fluid 1 in 100 in PBS or undiluted monoclonal culture supernatant) for 1 h at room temperature with shaking. This was followed by rinsing the membrane in distilled water and washing twice (10 min for each wash) in TBS containing 0.25% Tween (TTBS). In order to detect bound antibody, the membrane was incubated with sheep anti-mouse IgG - horse radish peroxidase conjugate (Scottish Antibody Production Unit, Lanarkshire, Scotland) diluted 1 in 200 in TTBS for 1 h at room temperature with shaking and subsequently washed twice for 10 min in TTBS. The nitrocellulose membrane was then incubated with freshly prepared substrate solution (30mg 4-chloro-1-napthol, 10ml methanol, 30 µl H_2O_2 in 50ml TBS) until dark blue bands appeared (approximately 10 min). The reaction was terminated by washing in excess distilled water.

2.7 Media and Medium Constituents Used for Monoclonal Antibody Production

2.7.1 Basic medium

Basic medium was prepared by adding the following constituents into 500 ml of sterile RPMI 1640 (Flow Laboratories Cat. No. 10-601-22).

12.5 ml	8% $NaHCO_3$ (BDH)
10.0 ml	0.5M Hepes (Sigma)
5.0 ml	0.1M Glutamine (Sigma)
4.0 ml	0.1M Na-Pyruvate (Gibco)

2.7.2 Fetal Calf Serum

Heat inactivated fetal calf serum (FCS-Gibco) was added into 500 ml basic medium in 50 ml, 75 ml and 100 ml volumes to obtain 10%, 15% and 20% FCS respectively.

2.7.3 HT and HAT media

HT and HAT media were made up, at least one week prior to use by adding 4 ml of the required 50 x stock solution (Gibco) into 200 ml of 20% FCS, and frozen pending sterility checks.

2.7.4 Sterility check

Basic medium, FCS, HT and HAT media were checked for sterility by adding 1 volume of the medium into 1 volume of nutrient broth in a sterile universal and incubating one week at 37°C and at room temperature.

2.7.5 Mixed Thymocyte Medium (MTM)

The method described by Reading (1982) was followed to prepare MTM. Two rats of different strains (e.g. Wistar, August) at 5-6 weeks of age were used. Their thymuses were removed aseptically and homogenized in basic medium to make a cell suspension. The cells were washed 3 times with basic medium and resuspended in 15% FCS medium and cultured at a cell density of 5.0×10^6 cells/ml for

36-48 h only. The medium was harvested by removing cells and debris by centrifugation at 4000g for 10 mins. Supernatants were dispensed into 10 ml volumes and stored at -20°C until used.

2.7.6 Polyethylene Glycol (PEG)

Polyethylene glycol (50%) was prepared by autoclaving 5g of crystalline PEG 4000 (BDH). The sterile molten PEG was allowed to cool to 37°C and then 5 ml of serum free RPMI at 37°C was added. The mixture was aliquoted in 1.2 ml volumes and stored in the dark at 4°C. Immediately prior to fusion PEG was warmed to 37°C and pH adjusted to between 8.5 and 9.0 with sterile 2M Tris/HCl.

2.7.7 Freezing Medium

Freezing medium was prepared by mixing 90% FCS with 10% fresh dimethyl sulphoxide (DMSO - Sigma) and kept frozen at -20°C until used.

2.7.8 Versene (EDTA in PBS) Stripping Solution

A 10 mM solution of EDTA (Sigma) was prepared in PBS into which 1% phenol red was added and the pH adjusted to between 7.1 and 7.3. The solution was sterilized through membrane filters (0.2 μm) and dispensed in 15 ml volumes.

CHAPTER 3

Expression of Outer Membrane Proteins by *Pasteurella multocida* Grown *in vivo* and *in vitro* Under Iron Restricted Conditions.

INTRODUCTION

SDS-PAGE analysis of the outer membrane proteins (OMPs) of *P. multocida* isolated from rabbits, turkeys, pigs and cattle grown in complex laboratory media and the recognition of certain major OMPs by immune sera has been reviewed in the general introduction.

Expression of iron-regulated proteins (IRP) by some Gram-negative bacteria and their possible role in the host immunity was also discussed.

In most studies iron restriction *in vitro* has been achieved by addition of the iron chelator 2,2'-dipyridyl into BHIB or NB (Griffiths *et al* 1985, Snipes *et al* 1988, Ikeda and Hirsh 1988, Donachie and Gilmour 1988).

The expression of IRPs with molecular masses of 96, 84 and 80 kDa by *P. multocida* of turkey origin grown in *in vitro* in turkey plasma and in iron-depleted BHIB was reported by Snipes *et al* (1988). When all sixteen serotypes of *P. multocida* (Heddleston's) were grown *in vitro* under iron restricted conditions an antigenically related IRP of molecular mass 84 kDa was produced (Ikeda and Hirsh 1988).

P. haemolytica, a closely related species to *P. multocida* was shown to produce several high molecular mass OMPs when grown *in vivo* and *in vitro* under iron restriction conditions (Donachie and Gilmour 1988). Recognition of the IRPs by antibodies present in

sera and lung washes of lambs recovering from pneumonic pasteurellosis, indicated the importance of incorporating these antigens in the development of new vaccines.

The experiments described here were, therefore, designed to study the OMP profile of *P. multocida* type A of bovine origin grown *in vivo* and *in vitro* under iron restricted conditions.

RESULTS

3.1 Growth of *P. multocida in vivo*

3.1.1 Growth in chambers implanted intraperitoneally in mice.

An attempt was made to grow *P. multocida* in silicon chambers [1.0 cm long silicon tubes sealed with 6 mm diameter, 0.45 µm filter membranes (Millipore, UK), on either end] implanted in the peritoneal cavity in mice. The cells recovered were 3.0×10^8 cfu in total from 20 chambers implanted intraperitoneally in 10 mice after an incubation period of 30 days with an initial bacterial count of 3.0×10^9 per chamber. As this number of bacteria was insufficient for the preparation of OMPs or vaccines the method was discontinued.

3.1.2 Growth in ascitic fluid of tumour induced mice

In order to obtain *in vivo* cells, *P. multocida* was grown in ascitic fluid of tumour induced mice. Tumour growth was promoted by inoculating plasmacytoma cells into mice which had been treated with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane - Sigma) three days previously. *P. multocida* 4.0×10^8 cfu in 0.5 ml of PBS were inoculated into ascitic fluid. The bacteria harvested

after 24h from 2 ml of ascitic fluid collected from six mice were 5.5×10^7 cfu and were insufficient to fulfill the above stated criterion.

3.1.3 Recovery of *P. multocida* from pleural effusion of sheep.

As an alternative method for the recovery of *in vivo P. multocida* cells, lambs were inoculated intrapleurally with 5.0 ml of a 6h broth culture (1.5×10^9 cfu/ml) between the 7th and 8th left ribs using a 19 gauge needle fitted onto a 5 ml syringe. Of the three lambs infected one was found dead 18-22h following inoculation. The other two lambs which survived were killed by anaesthesia with intravenous pentobarbitone and exsanguination. Pleural effusion (up to 300 ml/sheep) was collected into two sterile bottles containing 2 ml of 100 μM EDTA which prevented the formation of fibrin clots. After removal of large particles by coarse filtration through muslin the filtrate was centrifuged at 1000g for 15 min to deposit blood cells, cell debris and other host cells. Bacterial cells were then pelleted from the supernatant by centrifugation at 5000g for 20 min, washed 3 times in PBS before being resuspended in 10 ml of PBS. Cells in the suspension were counted by the dilution method of Miles and Misra (1938), and checked for purity by examining a smear stained with Giemsa-stain and by plating onto SBA. An average of 1.25×10^8 cfu/ml bacteria were present in the pleural fluid of the two lambs. Half of the cell suspension was used to prepare OMP by the method described in section 2.3.4 while the remainder was heat inactivated

and freeze-dried (section 2.3.1) in preparation for use as a vaccine.

3.2 Growth of P. multocida in vitro under iron restricted and iron replete conditions

For this study *P. multocida* were grown in the following media.

3.2.1 BHIB or NB

P. multocida strains were grown in either BHIB or NB for 18 h at 37°C and 40°C.

3.2.2 80% Horse serum

P. multocida was also grown in 80% horse serum (Gibco) with 20% PBS for 18h at 37°C as described by Donachie and Gilmour (1988).

3.2.3 BHIB depleted of iron

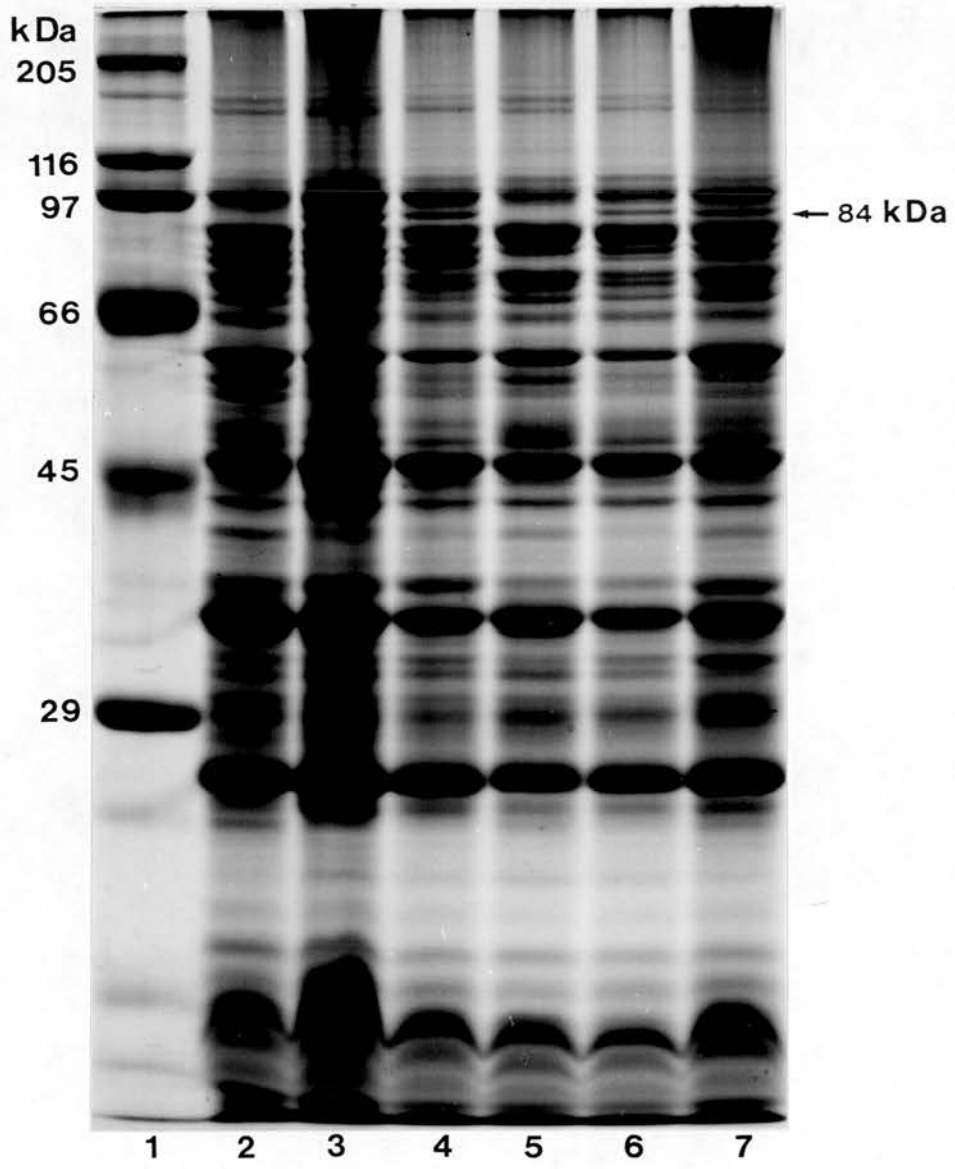
Bacteria were grown in 100 ml BHIB containing 2,2'-dipyridyl (BHIB 2,2'-dp) at 150 and 200 μM, at 37°C and 40°C for 18 h to optimise the concentration of 2,2'-dipyridyl and the growth temperature. Cells harvested by centrifugation at 5000g for 20 min were washed once in PBS before being resuspended in 2 ml distilled water. Samples (40 μl) were analysed by SDS-PAGE.

Determination of optimum concentration of 2,2'-dipyridyl and growth temperature by SDS-PAGE of whole cell preparations of *P. multocida* (A26)

Fig. 3.1 shows *P. multocida* whole cells separated on SDS-PAGE

Fig. 3.1. SDS-PAGE analysis of *P. multocida* (A26) whole cells. (Lane 1) Standards with molecular masses indicated in kDa; (Lane 2) whole cells grown in BHIB at 37°C; (Lane 3) BHIB 2,2'-dipyridyl (150 μM) at 37°C; (Lane 4) BHIB 2,2'-dipyridyl (200 μM) at 37°C; (Lane 5) BHIB at 40°C; (Lane 6) BHIB 2,2'-dipyridyl (150 μM) at 40°C; (Lane 7) BHIB 2,2'-dipyridyl (200 μM) at 40°C.

Fig. 3.1.



and stained with Coomasie blue. The major proteins with molecular masses of 25.0, 39.0, 47.0, 60.0, 77.0 and 97.0 kDa were present in all preparations. A high molecular mass protein of 84 kDa was present in whole cells grown in BHIB containing 2,2'-dipyridyl at 37°C and 40°C, but absent from those grown in BHIB, and was considered to be an iron-regulated OMP. This protein was expressed best in cultures containing 150 μM dipyridyl and grown at 37°C (Fig. 3.1, lane 3). These conditions were, therefore, adopted as the standard procedure.

Samples were analysed on 10% polyacrylamide gels and stained with Coomassie blue by the sequential staining method described by Hancock and Poxton (1988).

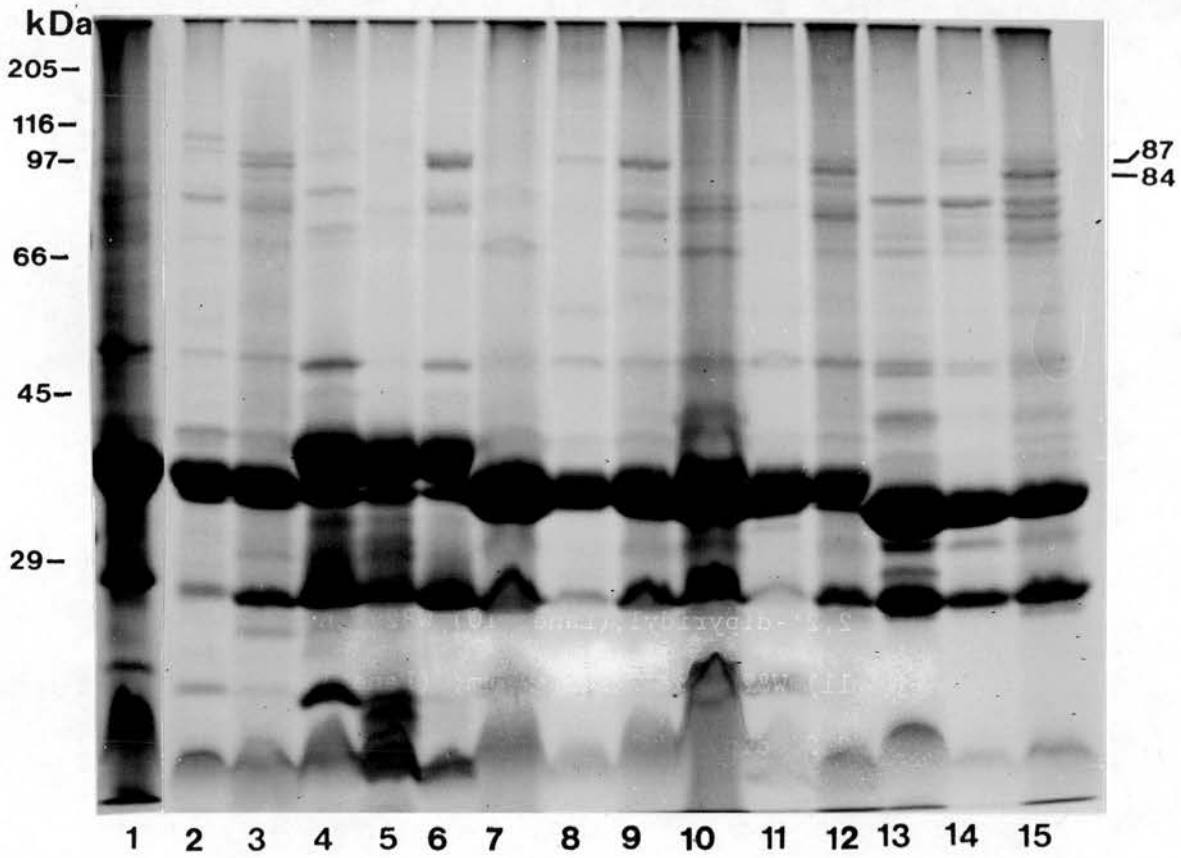
3.3 Analysis of OMP preparations of five isolates by SDS-PAGE

Five isolates of *P. multocida* (Strain 10322, W674, X200, W829, X120) selected randomly were grown in NB, NB containing 2,2'-dipyridyl and 80% horse serum. The OMPs prepared (see Section 2.3.4) were analysed by SDS-PAGE and the protein profile was compared.

Fig. 3.2 shows the results of SDS PAGE analysis of outer membrane preparations of 5 different isolates of *P. multocida* (Strain 10322, W674, X200, W829 and X120) grown in NB, 80% horse serum and NB containing 2,2'-dipyridyl. The high molecular mass OMP of 84 kDa observed previously in whole cell preparations (Fig. 3.1) was also present in the outer membrane preparations of all 5 isolates grown in NB containing 2,2'-dipyridyl, but was absent in those from horse serum and NB cultures. One other high molecular

Fig. 3.2. OM preparations of five isolates of *P. multocida* grown under different cultural conditions and separated on SDS-PAGE. Position of standards with molecular masses indicated in kDa; (Lane 1) W674 in NB; (Lane 2) W674 in horse serum; (Lane 3) W674 in NB 2,2'-dipyridyl; (Lane 4) reference 10322 in NB; (Lane 5) reference 10322 in horse serum; (Lane 6) reference 10322 in NB 2,2'-dipyridyl; (Lane 7) X200 in NB; (Lane 8) X200 in horse serum; (Lane 9) X200 in NB 2,2'-dipyridyl;(Lane 10) W829 in NB; (Lane 11) W829 in horse serum; (Lane 12) W829 in NB 2,2'-dipyridyl; (Lane 13) X120 in NB; (Lane 14) X120 in horse serum; (Lane 15) X120 in NB 2,2'-dipyridyl.

Fig. 3.2



mass protein of 87 kDa was evident in outer membrane preparations of dipyridyl grown cultures and in all except one (strain 10322) of horse serum grown cultures.

Variations in the OMP profile between isolates were observed when they were grown in horse serum. Two isolates (strain 10322 and W674) produced OMPs of 91 and 92.5 respectively, while in W829 and X120 a protein with molecular mass of 89 kDa was observed in horse serum grown cultures.

Major OMPs of 77 and those below 47 kDa were observed to be common to all isolates.

3.4 Comparison of outer membrane profile of *P. multocida* (A26) grown *in vitro* and *in vivo*

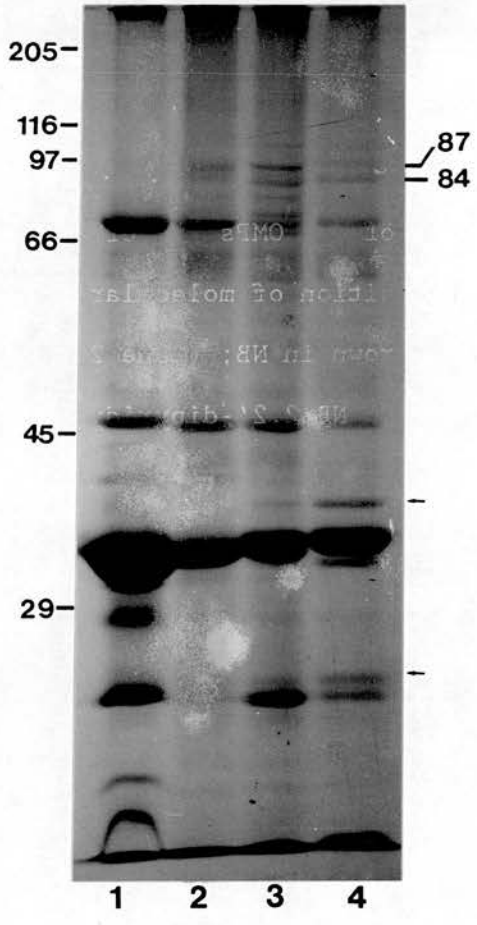
This strain was selected because of its low LD_{50} for use in immunization and challenge experiments (described in Chapter 4).

The protein expression of Strain A26 of *P. multocida* grown *in vivo* was compared with cells grown *in vitro* under iron restriction conditions as described in 3.3.

Fig. 3.3 shows the outer membrane protein profile of *P. multocida* (A26) grown *in vivo* and *in vitro* under various conditions. The protein profile observed here was very similar to that described previously with another five type A strains (fig. 3.2). The strain A26 when grown *in vivo* also expressed the 84 and 87 kDa proteins which were produced under iron-restricted conditions *in vitro*. Additionally, two other proteins with molecular masses of 36 and 22 kDa were observed to be unique to cells grown *in vivo* (Fig. 3.3).

Fig. 3.3. SDS-PAGE of OMPs of *P. multocida* (A26), position of molecular mass standards; (Lane 1) grown in NB; (Lane 2) in horse serum; (Lane 3) in NB 2,2′-dipyridyl and (Lane 4) in *in vivo*.

Fig. 3.3.



DISCUSSION

As iron restriction occurs naturally in an infection in the host, some bacteria adapt to this environment by producing siderophores and high molecular mass OMPs (Griffiths 1985; Overbeeke and Lugtenberg 1980). Production of siderophores by *P. multocida* has been reported by Hu *et al* (1986). The changes that may occur in the protein composition of the outer membrane of *P. multocida* when grown *in vitro* under iron restriction and *in vivo* are yet to be understood.

These experiments were designed to compare the expression of OMPs by *P. multocida* grown *in vitro* under iron restriction and *in vivo*, with that grown in iron rich medium.

The major OMPs observed in *in vitro* cultures in iron replete medium in this study were those with molecular masses of 21 or 25, 32, 33, 47 and 77 kDa as compared to those of 27, 37.5, 49.5, 58.7 and 64.4 kDa described by Lu *et al* (1988a). The heavy band in a doublet with an approximate molecular mass of 36 kDa observed by Lugtenberg *et al* (1986) was also evident in this study but with a slightly lower molecular mass of 33 kDa. Corbett *et al* (1984) described a matrix protein of 35 kDa found in an outer membrane preparation, KSCN extract and 2.5% NaCl extract and also in the ribosomal fraction of *P. multocida*. The differences in molecular masses of major proteins may have arisen due to the fact that they were not electrophoresed under identical conditions. The protein being referred to as 37.5, 36, 35 and 33 kDa, are very likely to be the same major OMP.

Cells of *P. multocida* grown under iron-depleted conditions expressed proteins of high molecular mass which were not present when the same culture was grown in NB or BHIB. These proteins were 87 and 84 kDa in molecular mass and were also found in cells recovered directly without subculture from the pleural fluid of lambs with experimentally induced pleural effusions. Only the 87 kDa protein was evident when bacteria were grown in horse serum (80%). In addition to 87 and 84 kDa proteins, two other proteins of 36 and 22 kDa were evident in *in vivo* grown cells.

Expression of a protein of 84 kDa by all the somatic types of *P. multocida* (except serotype 12) occurred when bacteria were grown in BHIB containing 2,2'-dipyridyl (Ikeda and Hirsh 1988). This is in agreement with the present findings, in that all six isolates examined have produced 84 kDa protein under iron restriction. When grown in turkey plasma at 41^{o}C, *P. multocida* of turkey origin expressed OMPs with molecular masses of 96, 84 and 80 kDa, which were also induced when the same strain was grown in BHIB with 2,2'-dipyridyl (Snipes *et al* 1988).

In this present study, the protein with 96 kDa molecular mass was not observed, but an 87 kDa protein was observed. Here *P. multocida* (A26) grown in NB with 2,2'-dipyridyl expressed an iron-regulated protein with molecular mass of 80 kDa. The differences seen in proteins expressed by *P. multocida* in this study may be due to the fact that the strains studied were bovine isolates which may produce different OMPs under iron restriction, compared to turkey isolates.

Although the role of these high molecular mass OMPs of *P. multocida* is not known, the involvement of similar proteins of other Gram-negative bacteria in iron acquisition is well documented (Sokol and Wood 1983; Griffiths 1985). Demonstration of antibodies to the iron-regulated proteins of other Gram-negative bacteria (Shand *et al* 1985; Griffiths *et al*; Bolin *et al* 1987; Donachie and Gilmour 1988) and the protective capacity of such antibodies (Bolin *et al* 1987) indicates the possible use of these proteins as candidate antigens in the formulation of new vaccines. Evaluation of the protective efficacy of vaccines containing *P. multocida* grown under different cultural conditions described in this study will be discussed in the next chapter.

CHAPTER 4

Evaluation of the Immune Response of Mice to *P. multocida* Grown *in vivo* and *in vitro* Under Iron Restricted Conditions

INTRODUCTION

The outer membrane protein (OMP) profile of *P. multocida* grown under various conditions was discussed in the previous chapter. The immunogenicity of heat killed *P. multocida* whole cells of bovine origin grown in iron replete medium, was studied in mice by Abdullahi (1987). Although antibodies to major OMPs were demonstrated in immune sera from mice, they were not related to the protection induced by *P. multocida* whole cell vaccines. The presence of an immunogenic OMP of 37.5 kDa expressed on the cell surface of *P. multocida* isolates from rabbits indicated its possible importance in immunity in rabbits (Lu *et al* 1988a and 1988b).

Since it is evident that *P. multocida* produce additional OMPs under iron restriction, the role of these proteins in the immune response of mice is an important aspect which needs to be investigated. Active immunization of mice with vaccines incorporating cells grown *in vivo* and *in vitro* under iron restriction and evaluation of the immune response of mice to these cells was therefore studied.

RESULTS

4.1. Selection of an isolate for vaccine experiments

Most bovine isolates of *P. multocida* type A were not very

virulent for mice with LD_{50} being in the order of 10^7-10^8 live organisms (Abdullahi 1987). In order to evaluate the immune response of *P. multocida* grown *in vitro* and *in vivo* in mice, a highly virulent strain was required. The virulence of four strains, in terms of LD_{50}, was assessed after two passages in mice.

4.1.1. Passage of *P. multocida* in mice

A six hour broth culture of each isolate (A33, A580, X109 and A26) was inoculated intraperitoneally in 0.2 ml volumes into individual mice. Blood (0.1 ml) collected via the inferior vena cava of dead mice was subsequently inoculated intraperitoneally into other mice. Heart blood from the second set of mice was plated onto SBA, and incubated at 37°C in a moist chamber. The cultures grown on SBA were used to assess the LD_{50}.

4.1.2. Determination of the LD_{50} of passaged cultures.

Table 4.1 indicates the LD_{50} values of passaged cultures assessed by the method described in section 2.4.2.

Strain A26 demonstrated the highest virulence (an LD_{50} of 6 organisms) and was selected to be used in the vaccine experiment.

4.2. Determination of virulence (LD_{50}) of strain A26 grown under various conditions

P. multocida when grown under various conditions expressed different outer membrane proteins. The effect of these proteins on the virulence of the organism was studied in order to determine whether virulence could be correlated with the ability of these

Table 4.1. LD_{50} titration of mouse passaged cultures

Strain No.	Colony counts of 6h broth cultures (cfu/ml)	LD_{50} in terms of titre	LD_{50} in terms of No. of organisms
X109	3.5×10^{9}	$10^{-8.69}$	7
A26	2.0×10^{9}	$10^{-8.5}$	6
A580	2.5×10^{9}	$10^{-7.39}$	102
A33	5.0×10^{7}	$10^{-3.37}$	2×10^{4}

proteins to elicit an antibody response. A LD_{50} study of *P. multocida* (A26) grown *in vitro* and *in vivo* was therefore performed. Ten-fold serial dilutions in peptone water were made from cultures of bacteria grown in NB, NB containing 2,2'-dipyridyl and 80% horse serum for 6h at 37°C. *In vivo* grown *P. multocida*, recovered from pleural fluid of lambs, were also diluted in a similar manner and the LD_{50} was determined as described in section 2.4.2. The number of viable bacteria in cell suspensions were counted by the method of Miles and Misra (1938). Table 4.2 indicates the LD_{50} values of cells grown under various conditions.

As indicated in the table 4.2, cells grown in NB and *in vivo* demonstrated the greatest virulence, while NB containing 2,2'-dipyridyl and horse serum grown cells were found to be the least virulent.

4.3. Immunisation of mice with bacteria grown in vitro under iron restricted conditions, and *in vivo*.

a) Bacterial strain

P. multocida A26 strain was used in this study.

b) Preparation of vaccines

Four vaccines each incorporating cells grown in either NB, horse serum and NB with 2,2'-dipyridyl grown, or *in vivo* cells (section 3.1 c and 3.2 a, b and c) were prepared as described in section 2.4.3 (a).

Table 4.2. LD_{50} values of *P. multocida* (A26) grown in various conditions

Growth conditions	Viable count cfu/ml	LD_{50} in terms of titre	LD_{50} in terms of No. of organisms
NB	a) 1.0×10^{10}	$10^{-5.19}$	7.0×10^{4}
	b) 2.75×10^{8}	$10^{-4.65}$	6.1×10^{3}
NB containing 2,2'-dipyridyl	a) 6.5×10^{9}	$10^{-1.1}$	5.1×10^{8}
	b) 4.0×10^{9}	$10^{-2.5}$	1.2×10^{7}
80% horse serum	a) 1.25×10^{10}	ND	ND
	b) 4.0×10^{10}	$10^{-2.49}$	1.2×10^{8}
In vivo	a) 5.0×10^{7}	$10^{-4.1}$	3.9×10^{3}
	b) 1.5×10^{8}	$10^{-4.53}$	4.4×10^{3}

a and b are replicates
ND - not determined

c) Mice

As described in section 2.4.1.

d) Immunization protocol

Each vaccine (0.2 ml) containing heat killed organism in adjuvant was inoculated subcutaneously into seventy-two mice on day 0 and day 14. An equal number of mice were allocated as unvaccinated controls.

4.3.1. Challenge of mice

a) Preparation of challenge inocula

The homologous strain (A26) was passaged twice in mice and used to prepare the challenge inocula. Two to three colonies from the passaged culture, from SBA plates, were inoculated into 10 ml of NB and grown at $37^{o}C$ for 6h with constant agitation. A series of doubling dilutions of the bacterial suspension (up to 27th dilution) was made in NB. The number of viable bacteria in the suspension was counted by the dilution method of Miles and Misra (1938) and was found to be 8.5×10^{9} cfu/ml.

b) Inoculation of mice

Three mice from each of the vaccinated and unvaccinated control groups were challenged intraperitoneally with 0.1 ml of varying doubling dilutions ranging from the 2^{-4} to the 2^{-27}, on day 28. Deaths were recorded for up to 48 h after challenge (Table 4.3).

4.3.2. Evaluation of the immune response of mice to vaccines incorporating heat killed organisms grown under various conditions.

The response of mice to each vaccine was measured by the means of the log protection and serological analysis.

a) Determination of LD_{50} and log protection afforded by each vaccine.

The LD_{50} for each group of mice was calculated by the method of Karber (1939). The log protection for each vaccine was expressed as follows:

Log protection obtained by the vaccine =

$$LD_{50} \text{ value for the control mice} \; - \; LD_{50} \text{ value for the vaccinated mice}$$

Table 4.3 documents the results obtained.

The vaccine containing whole cells grown in NB with 2,2'-dipyridyl produced the highest log protection (> 6.36), of the four vaccines tested in mice. The lowest protection (5.07) was afforded by the *in vivo* cell vaccine. Although this was the case, the differences between the log protections afforded by four vaccines were small, and not statistically significant.

The LD_{50} of the strain A26 in this experiment was found to be 330 organisms when compared to the earlier value of 6.

This experiment was repeated with a higher challenge. Each vaccine was inoculated into forty-five mice, according to the protocol described previously. The challenge doses ranged from

Table 4.3. Results of protection experiment in mice

Titration of challenge dose	No. of mice surviving after vaccination with HKO grown in				Controls
	NB	horse serum	NB + 2,2'-dipyridyl	in vivo	
$10^{-1.204}$ (2^4)	2/3	2/3	3/3	1/3	0/3
$10^{-1.505}$	2/3	3/3	3/3	1/3	0/3
$10^{-1.806}$	2/3	3/3	3/3	2/3	0/3
$10^{-2.107}$	3/3	3/3	3/3	3/3	0/3
$10^{-2.709}$	3/3	3/3	3/3	3/3	0/3
$10^{-3.010}$	3/3	3/3	3/3	3/3	0/3
$10^{-3.111}$	3/3	3/3	3/3	3/3	0/3
$10^{-3.612}$	3/3	3/3	3/3	3/3	0/3
$10^{-3.913}$	3/3	3/3	3/3	3/3	0/3
$10^{-4.214}$	3/3	3/3	3/3	3/3	0/3
$10^{-4.515}$	3/3	3/3	3/3	3/3	0/3
$10^{-4.816}$	3/3	3/3	3/3	3/3	0/3
$10^{-5.117}$	3/3	3/3	3/3	3/3	0/3
$10^{-5.418}$	3/3	3/3	3/3	3/3	0/3
$10^{-5.719}$	3/3	3/3	3/3	3/3	0/3
$10^{-6.020}$	3/3	3/3	3/3	3/3	0/3
$10^{-6.321}$	3/3	3/3	3/3	3/3	0/3
$10^{-6.622}$	3/3	3/3	3/3	3/3	2/3
$10^{-6.923}$	3/3	3/3	3/3	3/3	2/3
$10^{-7.224}$	3/3	3/3	3/3	3/3	3/3
$10^{-7.525}$	3/3	3/3	3/3	3/3	3/3
$10^{-7.826}$	3/3	3/3	3/3	3/3	3/3
$10^{-8.127}$	3/3	3/3	3/3	3/3	3/3
LD_{50} (titre)	$10^{-1.774}$	$10^{-1.037}$	ND	$10^{-2.334}$	$10^{-7.404}$
LD_{50} (No. organisms)	1.4×10^8	7.8×10^8	$>7.8 \times 10^8$	3.9×10^7	3.3×10^2
Log protection	5.63	6.36	>6.36	5.07	

ND - not determined

undiluted to 2^{-8} (1 in 256) of a doubling dilution series. Five mice from each group of vaccinated and unvaccinated controls were challenged with these dilutions; and observed for 48h. The 6h broth culture used for challenging mice contained 1.2×10^{10} cfu/ml.

One mouse each from the groups vaccinated with NB and NB containing 2,2'-dipyridyl grown cells survived the highest dilution (2^{-8}) of the challenge. All the other mice died following challenge. Therefore, no evaluation of the vaccines could be made.

b) Serology

(i) Sera

Prior to infection a pooled sample of serum from each group of mice was prepared as described in section 2.4.3 (c) and labelled as "pre-challenge sera". Surviving mice in each of the vaccinated and unvaccinated groups were killed 48h after infection by cervical dislocation and blood collected from the inferior vena cava. Serum was separated and stored at -20°C until analysis.

(ii) Indirect ELISA to detect the antibody response of mice immunized with *P. multocida* whole cells grown under various conditions.

Whole cell ELISA was carried out basically according to the method described in section 2.6.2 with a modification of the preparation of whole cell antigen in coating plates. *P. multocida* grown in 10 ml NB, 80% horse serum or NB containing 2,2'-dipyridyl for 6h at 37°C were washed once in PBS and resuspended to the same

Fig. 4.1. Antibody response of mice immunized with cells grown in NB (-●-), horse serum (-■-), NB containing 2,2'-dipyridyl (-◆-) and *in vivo* (-⊟-) against NB grown *P. multocida* (A26) cells determined by indirect ELISA. The _____ line indicates the response of the unvaccinated control mice.

Fig. 4.2. Antibody response of mice immunized with cells grown in NB (-●-), horse serum (-■-), NB containing 2,2'-dipyridyl (-◆-) and *in vivo* (-⊟-) against horse serum grown *P. multocida* (A26) cells determined by indirect ELISA. The _____ line indicates the response of the unvaccinated control mice.

Fig.4.1 Reactivity of mouse serum against nutrient broth grown P.multocida cells determined by Indirect ELISA

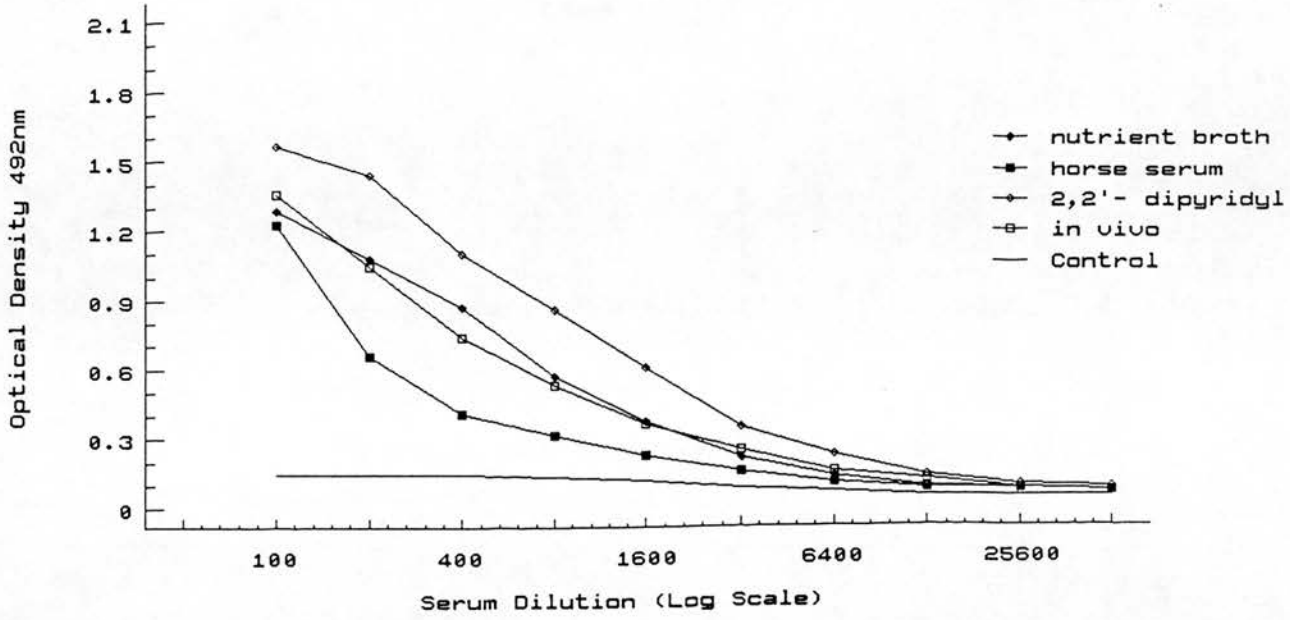


Fig.4.2 Reactivity of mouse serum against horse serum grown whole P.multocida cells determined by Indirect ELISA

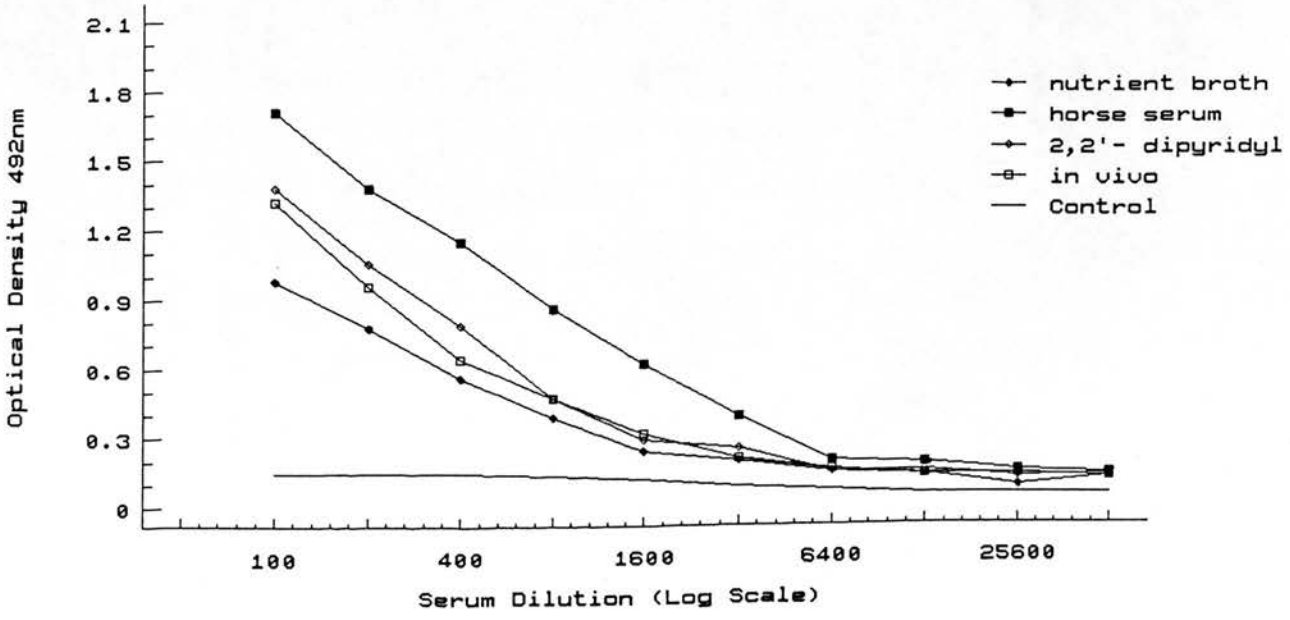


Fig. 4.3. Antibody response of mice immunized with cells grown in NB (-•-), horse serum (-■-), NB containing 2,2'-dipyridyl (-•-) and *in vivo* (-⊟-) against NB 2,2'-dipyridyl grown *P. multocida* (A26) cells determined by indirect ELISA. The _____ line indicates the response of the unvaccinated control mice.

Fig. 4.4. Antibody response of mice immunized with cells grown in NB (-•-), horse serum (-■-), NB containing 2,2'-dipyridyl (-•-) and *in vivo* (-⊟-) against *in vivo* grown *P. multocida* (A26) cells determined by indirect ELISA. The _____ line indicates the response of the unvaccinated control mice.

Fig.4.3 Reactivity of mouse serum against NB containing dipyridyl grown P.multocida cells determined by Indirect ELISA

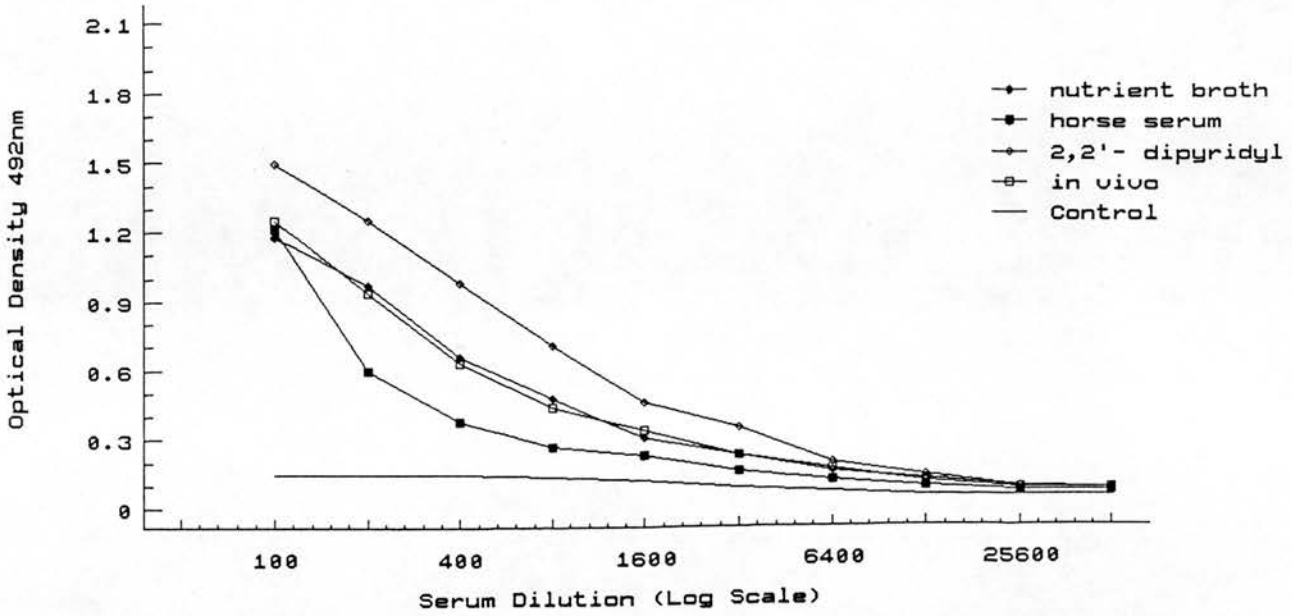
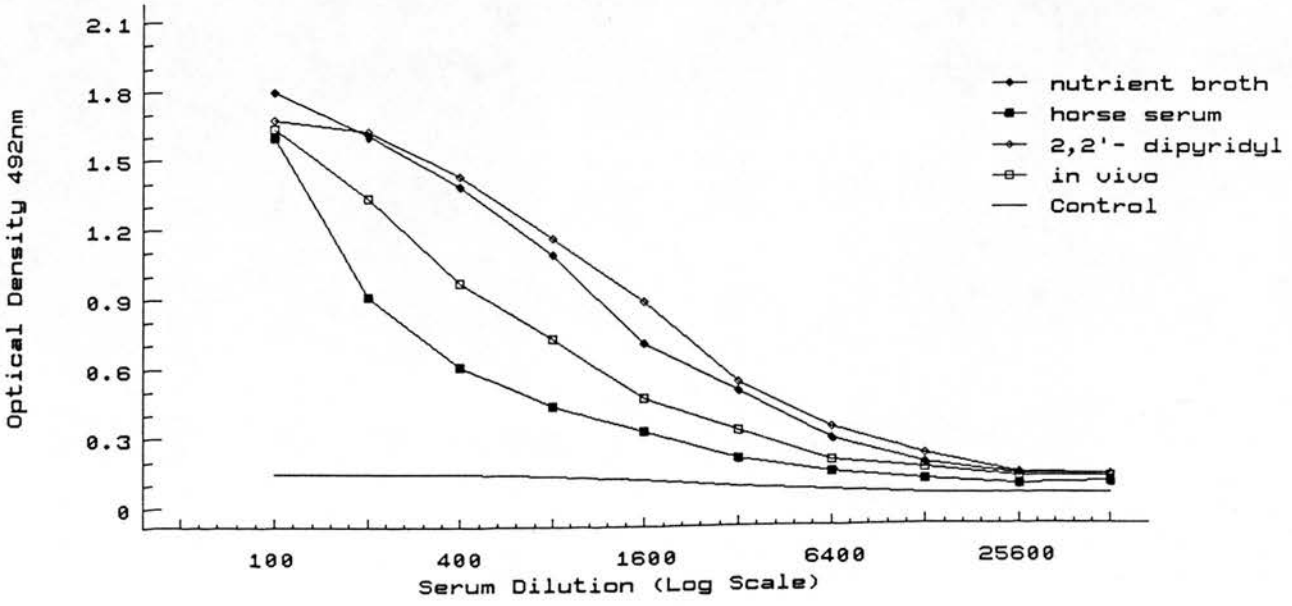


Fig.4.4 Reactivity of mouse serum against in vivo grown P.multocida cells determined by Indirect ELISA



volume in carbonate/bicarbonate buffer. Cells grown *in vivo* were also adjusted to the same concentration in carbonate/bicarbonate buffer after being washed once in PBS. In order to optimise the antigen and antibody concentration, a checkerboard titration was carried out with NB grown cells as antigen and mouse immune serum from chamber implanted mice as the standard positive (see appendix 1.1 for results). Each bacterial suspension (NB, horse serum, NB containing 2,2'-dipyridyl and *in vivo* grown) was coated onto a separate microtitre plate at the optimum concentration of antigen 10^7 cfu/ml, 100 µl/well.

After an initial dilution of 1 in 50, doubling dilutions (up to 2^{-10}) of sera (pre-challenge and post- challenge) from the four vaccinated groups and the unvaccinated control mice were made in PBS. These sera were tested against all four antigens by ELISA as described in section 2.6.2.

Fig. 4.1, 4.2, 4.3 and 4.4 show the ELISA results of pre-challenge immune response of mice to different whole cell antigens.

The antibody response induced by the vaccine consisting of NB 2,2'-dipyridyl grown cells was greater when compared to that induced by cells grown in NB, and *in vivo* (Fig. 4.1, 4.3 and 4.4). Serum of mice immunized with horse serum grown cells demonstrated the highest response only against the homologous cells and the lowest response against the cells grown under other conditions (Fig. 4.2). The response induced by the *in vivo* grown cells showed a lower but a similar pattern against all four types of cells.

(iii) Western blotting

Western blots of NB 2,2'-dipyridyl grown whole cells and OMP preparations of the strain A26 were prepared as in sections 2.6.3 and 2.6.4 and probed with sera (pre and post-challenge) at a 1 in 50 dilution. Antigen was transferred onto nitrocellulose paper, cut into strips and then probed with serum.

Fig. 4.5 and 4.6 demonstrate the reactivity of sera of vaccinated and unvaccinated control mice against separated OMP and whole cells of strain A26 respectively in Western blots. No antibodies were directed against the 84 and 87 kDa iron regulated proteins in sera of mice immunized with bacteria expressing these proteins (Lanes 3, 4, 8, 9 in Fig. 4.5). However, antibodies in sera of mice vaccinated with NB, NB containing 2,2'-dipyridyl grown cells reacted with 25, 33, 60, 77 and 97 kDa outer membrane proteins (Lanes 1, 3, 6 and 8 in Figs. 4.5, 4.6). Sera of mice immunized with horse serum grown cells recognised only the 25 and 33 kDa proteins. Sera from mice vaccinated with *in vivo* cells recognised proteins of molecular mass 25, 33 and 77 kDa.

DISCUSSION

The object of the experiments described was to study the importance of iron-regulated proteins expressed by *P. multocida* in the induction of immunity in mice.

For the purpose of challenging mice immunized with different vaccines, a strain with high virulence (low LD_{50}) was required as most of the strains of *P. multocida* type A were less virulent than

Fig. 4.5. Western blots of OMP of *P. multocida* (A26) grown in NB containing 2,2'-dipyridyl probed with sera of mice vaccinated with cells in 1 - NB, 2 - horse serum, 3 - NB 2,2'-dipyridyl, 4 - *in vivo* before challenge; 6 - 9 as above after challenge and 5 - unvaccinated.

Fig. 4.5.

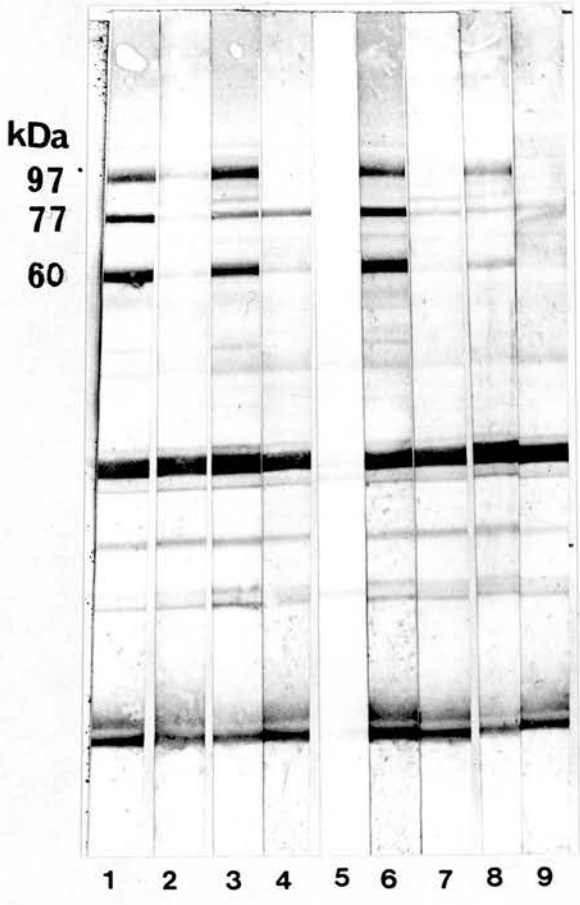
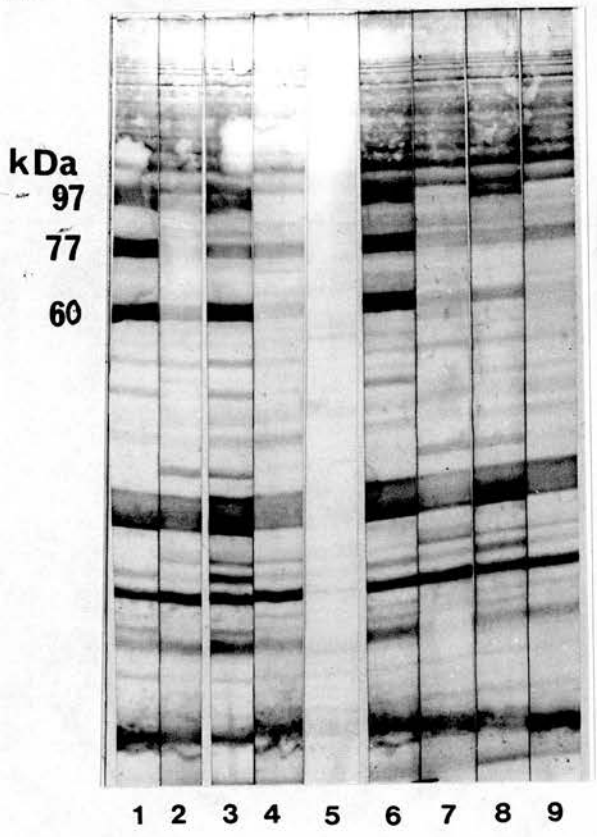


Fig. 4.6. Western blots of *P. multocida* (A26) whole cells grown in NB containing 2,2'-dipyridyl, probed with serum of mice vaccinated with cells grown in 1 - NB, 2 - horse serum, 3 - NB 2,2'-dipyridyl, 4 - *in vivo* before challenge; 6 - 9 as above after challenge and 5 - unvaccinated.

Fig. 4.6.



other serotypes for mice (Carter and Bigland 1958; Abdullahi 1987). After passage of four strains of *P. multocida* through mice to enhance their virulence, a strain with a low LD_{50} (6 organisms) was selected. However, the virulence of this strain changed in every experiment and affected the reproducibility of the experimental results. The use of a strain with stability in virulence is an essential criterion in vaccine experiments.

In mouse immunization experiments, although protection was demonstrated by each vaccine on challenge, a highly immunogenic vaccine, determined serologically, could not be identified. It is known that protection in mice can be achieved by immunization with whole cells of *P. multocida* (Collins 1973; Abdullahi 1987). The experiments described here, were planned in order to study the differences between the vaccines consisting of whole cells grown under various conditions for their protective ability. Due to the low number of mice used in the challenge in this experiment as a result of advice from a statistician, the protection afforded by each vaccine could not be statistically analysed. However, there was a suggestion that the vaccine comprising of cells grown in NB containing 2,2'-dipyridyl induced a greater protection than the others.

The antibody response of mice immunized with cells grown in NB containing 2,2'-dipyridyl was found to be greater than that of mice immunized with cells grown under other conditions, as determined by ELISA. Growth of *P. multocida* under iron restricted conditions *in vitro* may have produced other changes in antigenic profile than just the expression of iron regulated proteins on the bacterial

surface to which the mice responded. This view is supported by the appearance of two additional low molecular mass proteins recognised by sera from mice immunized with cells grown in NB containing 2,2'-dipyridyl, in Western blots (Fig. 4.6 - lane 3).

The high antibody response observed by ELISA against homologous cells in the sera of mice immunized with bacteria grown in horse serum suggests that these cells expressed different surface antigens than the cells grown in other ways. This was confirmed by the results of Western blots, where the least number of proteins of the dipyridyl grown cells were recognised by sera from mice immunized with cells grown in horse serum.

The failure to demonstrate antibodies to iron regulated proteins of 84 and 87 kDa may be because these proteins were not immunogenic in mice or they may not be exposed on the cell surface. The strong antibody response directed against a 37.5 kDa OMP observed in immune rabbit serum by Lu *et al* (1988a and 1988b) suggested that this protein was expressed on the cell surface and was easily accessible to the rabbit immune system. A similar response to the 33 kDa OMP in sera of all vaccinated mice, irrespective of the growth conditions, was demonstrated in this study. It is clear that these two proteins are probably the same antigen. Whether from the position of these proteins in the gels and immunoblots, they appear to be the same antigen. However, the differences in the molecular mass may have arisen as they were not electrophoresed under identical conditions.

Cells grown in NB and *in vivo* demonstrated the highest virulence. One could therefore, propose that cell surface

antigens or factors responsible for virulence could confer on these cells a greater immunogenicity. In this study no such relation between virulence and immunogenicity was established.

The results of these experiments have characterized some iron regulated proteins expressed by *P. multocida*. Although no response to these proteins was observed in mice, it will be important to investigate their role in immunity in cattle which may respond to these proteins differently.

CHAPTER 5

Effector Mechanisms of Immunity to *P. multocida* in Mice

INTRODUCTION

As has been previously discussed in Chapter 1, the mechanisms of immunity to *P. multocida* still remain unresolved. Resistance of type A *P. multocida* to phagocytosis (Maheswaran and Thies 1979; Rush *et al* 1981; Ryu *et al* 1984) and to serum killing, particularly of the clinical isolates of type A (Blau *et al* 1987) have been reported.

Rises in titres of bactericidal antibodies in mice by vaccinating with KSCN extract of *P. multocida* was shown by Mukkur (1980). However, the mechanism or mechanisms by which the vaccinated animals resist challenge and thereby survive has yet to be elucidated.

The objectives of this study were, therefore, to investigate the susceptibility of *P. multocida* type A to killing by serum, and to phagocytosis by mouse peritoneal macrophages, and the ability of protective vaccines to stimulate these mechanisms of immunity in mice.

RESULTS

P. multocida strain W674 was used in this study. The vaccine was prepared with heat killed organisms (HKO) of W674 grown in NB by the methods described in sections 2.3.1 and 2.4.3 (a).

5.1. Vaccination and Challenge of mice

Swiss white mice of either sex at 4-6 weeks of age were allocated randomly into four groups of fifteen (Table 5.1). Animals in groups two and three were vaccinated with 0.2 ml of HKO vaccine s.c. on days 0 and 14. Mice in groups one and three were challenged i.p. with a 10 LD_{50} of a 6h broth culture of W674 on day 28. The challenge inoculum contained 3.0 x 10^8 cfu/0.1 ml which was approximately the 10 LD_{50} for this strain as determined by Abdullahi (1987). Group four mice were included as untreated controls.

In this experiment all fifteen mice in group one died within 24h of challenge. In contrast, none of the vaccinated mice in group 3 died after challenge. The HKO vaccine, therefore, gave 100% protection against a challenge producing 100% mortality.

a) Immune mouse sera

The surviving mice were killed by placing them in a carbon dioxide chamber 48h after challenge. Blood from each mouse was collected from the inferior vena cava and serum was separated. The individual mouse serum samples were stored at -20°C until used.

5.2. Bactericidal assay

The assay was performed according to the method described by Sutherland (1988).

Table 5.1. Vaccination, challenge and time of bleeding of groups of mice.

	Vaccinated		Infected	Bled
Group	Day 0	Day 14	Day 28	Day 30
1	-	-	+	- *
2	+	+	-	+
3	+	+	+	+
4	-	-	-	+

* All mice died within 24 hours of infection
+ = treated
- = untreated

a) Preparation of bacterial inoculum

Two to three colonies removed from a SBA plate were inoculated into 10 ml NB and incubated for 18h at 37°C with shaking. A log phase culture was obtained by inoculating 100 µl from the 18h broth culture into another 10 ml NB and incubating for 3h at 37°C with shaking. Bacteria pelleted from 1 ml aliquots from 3h culture by centrifugation at 11,000 g for 5 min on a microfuge, were washed 3 times by centrifugation in modified barbitol buffer (MBB) (Oxoid, UK). After the final wash, bacteria were resuspended in 1 ml of MBB and tenfold serial dilutions were made in the same buffer. The aggregated colonies were dispersed by placing the bacterial suspensions in a sonicating waterbath for 10 min at 37°C. These bacterial suspensions were used as assay inocula in the optimisation of this test. Bacterial counts on these suspensions were made by the method of Miles and Misra (1938).

b) Standard positive serum

Hyperimmune serum against strain W674 produced (in rabbits) by the method described in section 2.5 was used as the standard positive serum.

c) Standard negative serum

Fetal bovine serum (FBS) tested and proved to be free of bactericidal antibody to *P. multocida* was used as a negative control.

The standard sera and test samples were heat- inactivated at 56°C for 30 min before assay to destroy endogenous complement.

d) Source of complement

Sera from new born calves or gnotobiotic lambs were tested for adequacy of complement activity by their ability to cause 100% killing of bacteria in the presence of the standard positive serum, and the absence of bactericidal antibodies. Complement was stored at -70°C until used.

5.2.1. Optimisation of bactericidal assay

Initially, optimal conditions for the demonstration of antibody mediated complement-dependent killing of *P. multocida* were determined with respect to bacterial concentration, incubation period and concentration of standard sera.

Standard sera (20 µl) were added in triplicate to wells of sterile, tissue culture grade microtitre plates (NUNC, Denmark). *P. multocida* bacterial inoculum (100 µl) in MBB was added to each triplicate assay well and plates were incubated at room temperature for 10 min. The complement source (80 µl) was then added to each well, (except when evaluating the effect of antibody on *P. multocida* in the absence of complement and the effect of complement alone on *P. multocida*, when 80 µl of heat-inactivated complement and 100 µl fresh complement were added respectively).

Plates were sealed with plastic film (Flow Laboratories, Irvine, UK) and incubated at 37°C. Volumes (10 µl) of each assay suspension were then removed with a multichannel pipette and inoculated on 12 x 12 cm square (Sterilin Ltd, Feltham, UK) SBA plates. When bacterial counts were too high for direct plate counting, as when the optimisation of the bacterial inoculum was

being carried out, 1 in 10 and 1 in 100 dilutions of each suspension were made in MBB diluent in microtitre plates before direct plating. The SBA plates were allowed to dry and then incubated at 37°C overnight.

The initial (T_o) bacterial count in each assay suspension was calculated as 0.5 x the mean cfu/ml determined in the assay inoculum. The mean cfu/ml of assay suspension remaining after 30 min (T_{30}) was calculated from the mean number of colonies counted in each triplicate 10 µl volume. The mean percentage of inoculum killed (%) of each serum sample was then calculated from the formula: $K = 1-[\text{mean cfu/ml at } T_{30} / \text{ mean cfu/ml at } T_o] \times 100$.

In this *in vitro* assay very little variations in counts between sample triplicates were found. The standard positive and negative sera gave reproducible results i.e. 100 and 0% K, respectively at every test.

The standard positive serum gave 100% killing of bacterial suspensions containing $\leq 1.06 \times 10^3$ cfu/ml. Killing dropped to 97.5% and 80.9% when the suspension titre was 1.06×10^4 and 1.06×10^5 cfu/ml respectively and no killing was observed when the suspension titre was $> 1.06 \times 10^6$ cfu/ml.

The optimal bacterial inoculum was therefore selected as 2.0×10^3 cfu/ml since this was totally killed by the standard positive serum and gave the maximum number of colonies which could be counted readily.

The 100% killing of *P. multocida* by the standard positive serum occurred only in the presence of complement. Any dilution of heat-inactivation of the complement source abolished its activity completely.

The standard negative serum plus complement was not bactericidal at any dilution, while the standard positive serum remained up to 90.6% bactericidal from 1 in 2 to a 1 in 16 dilution. At higher dilutions, activity dropped rapidly from 81.2% to 21.4% at 1 in 32 and 1 in 64 dilutions respectively.

After 2 min incubation only 2.6% killing occurred in the presence of the standard positive serum. At 10 and 20 min incubation 84.3 and 93.8% killing occurred. At and beyond 30 min the percentage killing was 100, and an incubation period of 30 min was therefore selected as optimal.

Optimal conditions for assay of bacterial killing of *P. multocida* were therefore found to be 20 µl of undiluted test serum with 100 µl of 2×10^3 cfu/ml of bacteria, mixed with 80 µl of undiluted fresh complement and incubation at $37^{\circ}C$ for 30 min.

5.2.2. Determination of bactericidal activity of immune mouse sera

Employing the optimal conditions described above, the assay was performed with individual mouse serum samples. In order to adjust the assay inoculum to 2.0×10^3 cfu/ml, a standard curve of cfu/ml against optical density of the bacterial suspensions at 420 nm was initially used. A variation of one log unit in the colony count occurred, affecting the efficiency of the assay. *P. multocida* when grown in NB for 6h at $37^{\circ}C$ gave a colony count of 5.0×10^9 cfu/ml consistently. Similarly, a log phase culture grown for 3h at $37^{\circ}C$ as described previously, resulted in $3.0 - 5.0 \times 10^8$ cfu/ml. Based on this criterion, the bacterial inoculum was diluted with

MBB to 2.0 x 10^3 cfu/ml thereby introducing very little variation in the count in the assay.

Bactericidal capacities of sera from groups 2, 3 and 4 are indicated in Table 5.2. The results will be interpreted later in the text.

5.3. Determination of antibody response of mice to *P. multocida* by indirect ELISA

Antibodies to *P. multocida* in mouse serum samples were detected by ELISA. *P. multocida* cell suspension used as the antigen was diluted 1 in 100 in carbonate/bicarbonate buffer pH 9.6, while each serum sample was diluted 1 in 100 in PBS/Tween. Table 5.2 indicates the ELISA titres of sera from groups 2, 3 and 4.

5.4. Statistical analysis

The Mann Whitney ranking test was used to determine significant differences between the groups, while the data were correlated by the Spearman-Jackson rank correlation test.

5.4.1. Analysis of immune mouse sera for the bactericidal activity and ELISA titres

The mean bactericidal capacities and ELISA titres of groups 2 and 3 (Table 5.2) were not significantly different from each other. Both groups had raised serum bactericidal capacity and ELISA titres compared to the unvaccinated (group 4) mice which had no bactericidal capacity and significantly lower ELISA titres ($P < 0.001$).

Table 5.2. Bactericidal capacities (% K) and ELISA optical densities of immune mouse sera

Animal No.	Group 2 (Vaccinated only)		Group 3 (Vaccinated and challenged)		Group 4 (Unvaccinated unchallenged)	
	%K*	Elisa OD	%K	Elisa OD	%K	Elisa OD
1	NA	NA	NA	NA	0	0.000
2	4	0.242	70	0.148	0	0.000
3	60	0.200	44	0.181	0	0.032
4	70	0.220	44	0.148	0	0.026
5	84	0.127	74	0.259	0	0.017
6	74	0.205	80	0.222	0	0.006
7	80	0.155	60	NA	0	0.017
8	84	0.166	84	0.337	0	0.007
9	77	0.222	94	0.306	0	0.001
10	57	0.142	77	NA	0	0.000
11	84	0.106	74	NA	0	0.000
12	60	0.175	87	0.249	0	0.000
13	57	0.197	54	0.113	0	0.053
14	50	0.219	97	0.119	0	0.039
15	60	0.199	70	0.151	0	0.054
group mean (±S.E.)	64.4 (±5.6)	0.1839 (±0.04)	72.1 (±4.5)	0.2030 (±0.08)	0	0.0168 (±0.02)

NA - Not available

* %K = 1 - [mean cfu ml^{-1} at T_{30}/mean cfu ml^{-1} at To] x 100

Bactericidal capacity of serum samples correlated well with ELISA titres ($P < 0.001$).

5.5. Phagocytosis assay

This was performed in collaboration with A.D. Sutherland, employing the optimal conditions described for *P. haemolytica* by Sutherland (1989, Thesis in preparation).

5.5.1. Bacterial inoculum

P. multocida (W674) inoculum for the assay was prepared from an 18h 3H-thymidine labelled culture and adjusted to the required concentration as described by Sutherland (1989). The bacterial count in the suspension was confirmed by the dilution method of Miles and Misra (1938).

5.5.2. Decapsulation of *P. multocida*

The method described by Maheswaran and Thies (1979) was followed, for the decapsulation of *P. multocida*. 3H- thymidine labelled *P. multocida* W674, pelleted from a 1 ml sample of 18h broth culture was resuspended in 1 ml of PBS at pH 6.0. The bacterial suspension in PBS was mixed with 1 ml of PBS (pH 6.0) containing 5000 National Formulary Units of testicular hyaluronidase (Sigma) and incubated at 37^{o}C for 3h. Decapsulated bacteria were removed by centrifugation at 11,000g for 5 min, washed three times in Hank's Balanced Salt Solution (HBSS) and resuspended in "Hanks medium" to the required concentration. The Hanks medium was prepared by supplementing HBSS with 10%

heat-inactivated fetal bovine serum, 2% 30 mM Hepes buffer, 1% sodium bicarbonate and adjusted to pH 7.2 with 1M NaOH. The number of bacteria in the suspension was confirmed by retrospective plate counting on SBA.

5.5.3. Recovery of mouse peritoneal macrophages

Six mice (Swiss white/Balb/c crosses) were each inoculated intraperitoneally with 2.0 ml of Bayol (Esso). The mice were killed by placing them in a carbon dioxide chamber 48h after inoculation, and the peritoneal cavities were washed several times with HBSS after opening the abdominal walls. The HBSS containing peritoneal macrophages was collected by sterile pipettes into sterile universal bottles. The cells recovered by sedimentation at 100g for 10 min at room temperature, were washed three times with HBSS and finally resuspended in 1 ml of Hanks medium. An aliquot (10 µl) of cells was added into 90 ul of Trypan blue dye (BDH) and the number of viable macrophages and the total number of cells were counted in an improved Neubauer cell counting chamber. The peritoneal macrophages recovered by this way were 96% viable and consisted of greater than 80% of the cell population as assessed by morphological criteria. The number of macrophages recovered were 2.25×10^7 in the total volume of 1.5 ml, and was adjusted to give 2.5×10^6 cells/ml.

5.5.4. Sera tested in the phagocytosis assay

a) Standard positive serum

Sera from mice implanted with chambers containing live *P.*

multocida (Section 3.1.a) were used as the standard positive serum in this assay. Blood was collected from mice 30 days after the implantation of chambers and the serum was separated and stored at -20°C until used.

b) Standard negative serum

Sera from normal, healthy, six week old mice were used as standard negative serum.

c) Immune mouse serum

Most of the serum samples in groups 2, 3 and 4 (Section 5.1, Table 5.1) were not available in sufficient volumes to be tested individually for phagocytic activity. Pooled serum samples were therefore made from each group by mixing the equal numbers of serum samples to give equal final volumes.

All the serum samples were heat-inactivated at 56°C for 30 min before use.

5.5.5. Determination of the ability of immune mouse sera to enhance phagocytosis of *P. multocida* by mouse peritoneal macrophages

This was performed essentially according to the method described by Sutherland (1989 Thesis in preparation).

In order to study the effect of decapsulation of *P. multocida* on phagocytosis, the assay was carried out under the conditions described below, with hyaluronidase treated bacterial suspensions.

The assay was performed in microtitre SV filtration plates (Millipore, USA) using the Titertek vacuum apparatus (Millipore, USA).

The microtitre plate filter membranes with a pore size 5.0 μM were wetted by the addition of 10 μl of Hanks medium to each assay well. The bacterial suspension in 100 μl volumes were added into each assay well except for three blank control wells which received 120 μl of Hanks medium. At the same time the bacterial suspension was also added in triplicate in 100 μl volumes to 2 ml of Scintilator 299 (Packard Instrument Co, USA) in plastic vials. These were counted as total activity controls on a B-isotope counter (Canberra Packard, U.K.).

The test sera and positive and negative control sera were then added in 20 μl volumes into triplicate assay wells. The plates were sealed with sterile plastic films and incubated at 37°C for 30 min to allow opsonisation to occur. The suspension of mouse peritoneal macrophages was then added in 80 μl volumes into each assay well except the bacteria only control wells which were inoculated with 80 μl of Hanks medium. Plates were again covered with sterile plastic film and incubated at 37°C for 30 min to allow phagocytosis to occur.

Bacteria that were not engulfed by peritoneal macrophages were removed by suction placing assay plates on the Titertek vacuum apparatus. This was followed by washing each well five times with 200 μl of HBSS. The retained peritoneal macrophages containing engulfed bacteria were dried onto membrane filters by placing assay plates at 37°C overnight in an incubator. Individual well filter

membranes were punched out and added into 2 ml of scintillant in plastic vials. Activity was counted on a B-isotope counter.

In this assay *P. multocida* bacterial suspensions, both capsulated and decapsulated,contained 3.0×10^7 cfu/ml, giving a ratio of 15:1 of bacteria to macrophages.

The percentage of bacteria in the suspension that were phagocytosed was calculated from the formula:

$$\% \text{ phagocytosis} = 100 \times \frac{\text{The mean counts per min (cpm) in test sample - the cpm in bacteria only}}{\text{The mean cpm in the bacterial inoculum - the cpm in bacteria only}}$$

Table 5.3 indicates the percentage phagocytosis in each pooled immune mouse serum sample. The percentage phagocytosis in sera of mice which were challenged after vaccination (group 3) was slightly higher than that of mice which were vaccinated only (group 2). Decapsulation of bacteria by treatment with hyaluronidase seemed to have no effect on the % phagocytosis. The % observed in the bacteria only and the blank controls ensured that the extracellular bacteria had been removed from assay suspensions. The low percentages of phagocytosis in this study may have resulted from the high ratio of bacteria to macrophages.

DISCUSSION

A vaccine of heat-killed organisms (HKO) of *P. multocida* type A induced 100% protection in mice against a homologous challenge of

Table 5.3. The ability of immune mouse serum to enhance phagocytosis of *P. multocida* (% phagocytosis) by mouse peritoneal macrophages

Serum	% phagocytosis of capsulated *P. multocida*	% phagocytosis of decapsulated *P. multocida*
Pooled sample from group 2 (vaccinated only)	1.7	1.0
Pooled sample from group 3 (vaccinated and challenged)	12.4	8.4
Pooled sample from group 4 (unvaccinated, unchallenged)	0.2	0
Standard positive	5.3	6.9
Standard negative	0	0.5

10 LD_{50}, which gave 100% mortality in the unvaccinated control group. Although *P. multocida* is very virulent for mice, excellent protection against parenteral infection was attained by others (Woolcock and Collins 1976) using injections of HKO vaccine incorporated into Freund's complete adjuvant. Humoral immunity and the possible involvement of polymorphonuclear leucocytes has been implicated in protection of effectively immunized mice (Collins and Woolcock 1976), but the mechanisms of immunity were not examined. The experiments described here were designed to study the mechanisms of immunity by which immune mouse serum overcomes infection with particular reference to mechanisms such as the complement-mediated, antibody-dependent killing of bacteria and the augmentation of phagocytosis by mouse peritoneal macrophages.

In this study protection against *P. multocida* was clearly associated with bactericidal antibodies produced by vaccination. Unvaccinated, susceptible control mice had no bactericidal antibodies while vaccinated protected mice did have bactericidal antibodies.

During the optimisation of the bactericidal assay both complement and specific antibodies were found to be essential components indicating that this activity was operating via the classical pathway of complement activation and that in the absence of antibody the alternative pathway was not activated. Antibody-mediated killing by the classical pathway of complement therefore appears to be an important mechanism of immunity against *P. multocida* and can be stimulated by vaccination.

The results also suggested that immune mouse serum was capable of enhancing phagocytosis of *P. multocida* by peritoneal macrophages. Absence of any phagocytosis with non-immune serum and the enhanced phagocytosis by immune serum confirmed the role of specific antibodies as opsonins in the process of phagocytosis. The slightly higher percentage phagocytosis observed in the serum of mice which were challenged may possibly be due to an anamnestic response within 48h or may be due to a unique response towards the virulent organisms. The response induced against the live organisms during the 48h after challenge may be an absolute requirement for the activation of the macrophages and/or the opsonization of bacteria by specific antibodies which enhanced phagocytosis.

The assay had to be performed with pooled serum samples because of the non-availability of certain individual mouse serum samples. Significant differences between groups and correlations of data could not be evaluated because of the small sample size. It was clear, however, that vaccination induced antibodies which enhanced phagocytosis but whether this correlated with protection could not be studied.

Anti-phagocytic activity of the hyaluronic acid capsule of *P. multocida* was reported by Maheswaran and Thies (1979) and Anderson *et al* (1984). Removal of this capsule might therefore, make the bacteria more susceptible to phagocytosis. In this study, decapsulation of bacteria by hyaluronidase treatment did not influence the phagocytosis revealing that the hyaluronic acid capsule in fact was not anti-phagocytic. Consistent with this

observation was a report by Ryu *et al* (1984) that a component of the capsule other than the hyaluronic acid was responsible for the anti-phagocytic effect of *P. multocida*. However, without proving that hyaluronidase treatment removed the capsular hyaluronic acid completely, it is not possible to rule out its anti-phagocytic effect.

The reported ability of non-immune serum to opsonize rabbit isolates of *P. multocida* as effectively as immune serum indicates that non-specific opsonins, such as cross- reacting antibodies may play a role in enhancing phagocytosis (Hofing *et al* 1979, Rush *et al* 1981). In this context, the involvement of the specific antibodies in the control of *P. multocida* infection was unclear. Resistance of *P. multocida* type D rabbit isolates to polymorphonuclear neutrophil phagocytosis (Anderson *et al* 1984, Rush 1989) suggested the involvement of immune mechanisms other than phagocytosis in combatting the virulent organism.

That cell-mediated immunity (CMI) was also involved in protection can not be discounted, but Carter (1967), Woolcock and Collins (1976) and Collins and Woolcock (1976) have all indicated that protection of mice with *P. multocida* vaccines is due to humoral immunity alone and classical CMI was therefore not investigated in this present study.

The results of this study suggest that in mice, the complement-mediated antibody-dependent killing of *P. multocida* is an important mechanism of immunity. It may be worthwhile investigating whether HKO vaccines stimulate bactericidal antibodies in cattle and whether the stimulation of these antibodies correlates with immunity.

The bactericidal capacity of sera correlated ($P < 0.001$) with their ELISA titres to whole-cell antigen suggesting that this rapid simple ELISA may be useful in screening vaccinated animals for protective antibody. The correlation of bactericidal capacity with whole-cell ELISA titres suggested that cell-surface antigens were involved in stimulating bactericidal antibodies. Examining the ability of individual cell-surface antigens to raise bactericidal antibodies may result in a more efficacious, single-antigen vaccine than HKO.

CHAPTER 6

Production and Characterization of Monoclonal Antibodies Against *Pasteurella multocida* type A.

INTRODUCTION

The immune response of *P. multocida* in mice is directed primarily against components of the cell surface i.e. capsule, LPS and OMP. Attempts at identifying single protective antigens employing mouse immune serum were unsuccessful due to the polyclonal nature of the serum.

One possible way to overcome this problem is by producing monoclonal antibodies (mAb) against cell surface antigens and evaluating the significance of such mAbs in immunity to *P. multocida*. Whether the mAb protects mice can then be determined by a passive mouse protection test (Moreno *et al*, 1983). If any mAb appears to protect mice, it is important to identify the mechanism by which the antibody mediates immunity. By binding to a cell surface component, antibody can enhance phagocytosis, fix complement resulting in lysis or disrupt the metabolic processes of bacteria resulting in death (Penaredondo *et al* 1988; Sutherland 1988; Coulton 1982).

Monoclonal antibodies can also be used for various other purposes. They have been employed for serotyping (Lam *et al* 1987) and for purification of antigens by affinity chromatography (Staehelin *et al*, 1981). In a sandwich or capture ELISA the specific antigen is captured by the mAb precoated onto the wells. This technique can be used to detect a specific antigen in a

suspected specimen (Hancock and Poxton 1988) or to measure the antibody status of animals to a particular organism (Shankarappa *et al* 1989a). Furthermore, it can be employed as a tool to evaluate cross-protection between strains or related species of bacteria (Lu *et al* 1988).

In this present study, the aim was to raise mAbs against *P. multocida* cell surface components. Upon characterization, their immunological mechanisms were investigated.

Mechanisms of hybridoma production

Spleen cells from mice immunized with antigen are fused with plasmacytoma cells in the presence of polyethylene glycol (PEG). Spleen cells die in tissue culture medium. Plamsacytoma cells which are defective in the enzyme hypoxanthine guanine phosphoribosyltransferase (HGPRT) die in the tissue culture medium supplemented with hypoxanthine, aminopterin and thymidine (HAT). Aminopterin blocks the synthesis of purines and pyrimidines. Only spleen cell/plasmacytoma hybrids survive in HAT medium. The contribution of the enzyme HGPRT from spleen cells enable the hybrids to utilize extraneous hypoxanthine to synthesise purines. Adding thymidine to culture medium makes it possible for hybrids to synthesise thymidine 5'-monophosphate (TMP) utilizing thymidine kinase (TK).

RESULTS

Part 1 - Production and characterization of monoclonal antibodies against *P. multocida* type A.

6.1. Production of monoclonal antibodies

6.1.1. Bacterial strain

P. multocida type A (W674) was used to immunize mice.

6.1.2. Preparation of immunizing agent

P. multocida grown overnight at 37°C in nutrient broth containing 2,2'-dipyridyl, were washed once in PBS and divided into 2 aliquots. One aliquot was resuspended in PBS to give a bacterial count of 8.0×10^9 cfu/ml. The remaining aliquot was resuspended in distilled water to contain 1.0×10^9 cfu/ml (vaccine 2). Both cell suspensions were killed by heating at 60°C for 90 min. The cell suspension in PBS was emulsified with an equal volume of Bayol/Arlacel. One part of the emulsion was then mixed with 3 parts of 1 in 10 alhydrogel (vaccine 1). The final inoculum of both vaccines therefore, contained 10^8 cfu/0.1 ml.

6.1.3. Mice

Six weeks old female Balb/c mice were used

6.1.4. Immunization Protocols

Two groups of five mice were each immunized with vaccine 1 or with 2 according to the following protocols.

a) Protocol 1

Day 0 - 0.1 ml vaccine 1 was inoculated intraperitoneally into 5 mice.

Day 14 - 0.1 ml vaccine 1 was inoculated intraperitoneally

Day 22 - Test bleed - Blood was collected from the tail vein

b) Protocol 2

Day 1 - 0.1 ml vaccine 2 was inoculated intraperitoneally into 5 mice.

The inoculations were repeated on days 2, 3, 8, 9, 10 and 22.

A test bleed was performed on day 29.

6.1.5. The immune response of mice to vaccines 1 and 2

The antibody response of each mouse was determined by whole cell ELISA (section 4.3.2.b ii and is indicated in figs 6.1.A and B). Doubling dilutions of serum were tested against NB 2,2'-dipyridyl grown cells.

Mouse no. 5 which had the highest antibody titre was selected for fusion and was boosted with 10^8 heat killed organisms in 0.1 ml saline intravenously 3 days prior to fusion.

6.1.6. Preparation of spleen cells for fusion

The mouse was sacrificed 3 days following intravenous boost and the spleen removed asceptically into 6 ml RPMI 1640 medium. The spleen was homogenized and the resulting cell suspension was washed three times by centrifugation at 450 g for 10 min, with RPMI 1640 medium and resuspended in 10 ml of basic medium (section 2.7.1.).

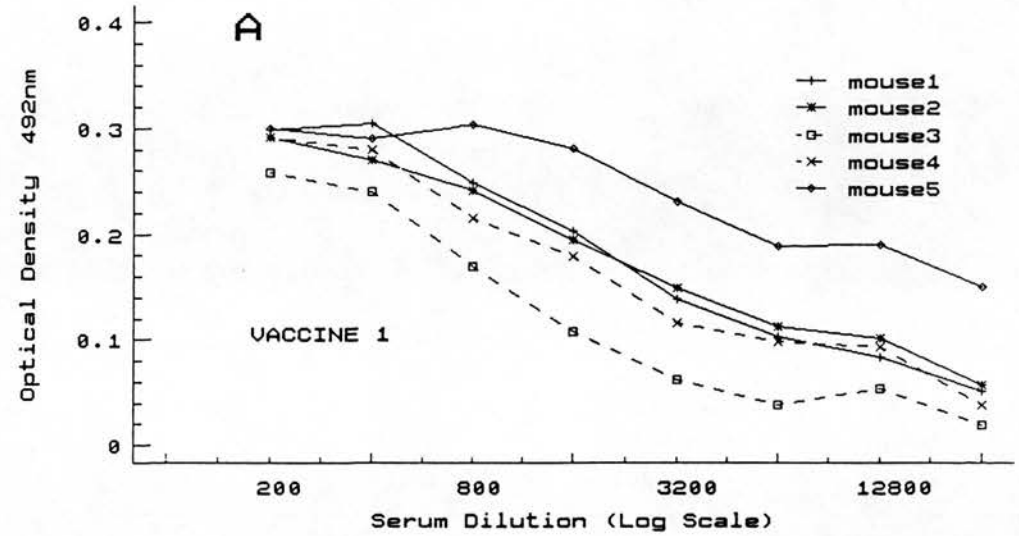
Fig. 6.1. Immune response of mice to vaccines 1 and 2 as determined by indirect ELISA

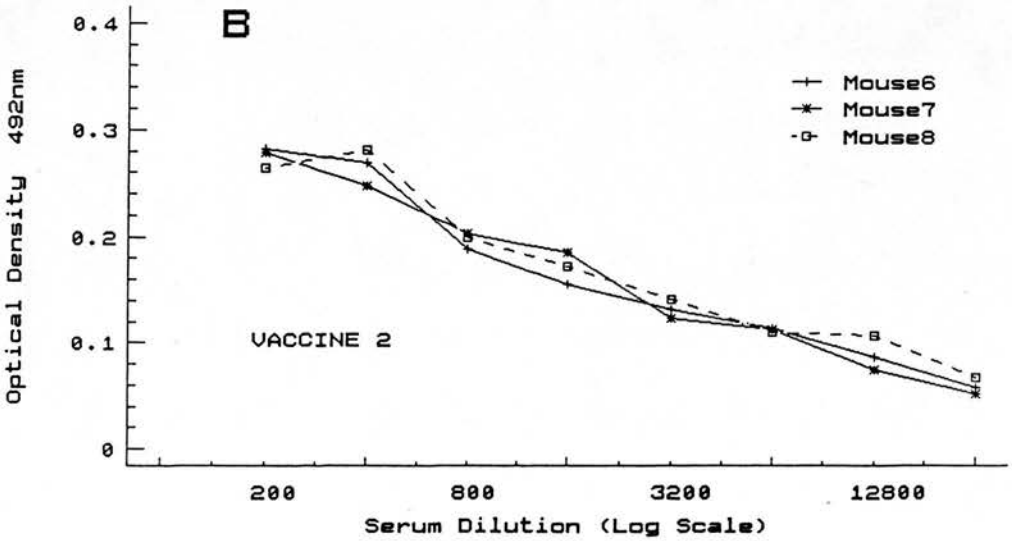
A) Response of mice to vaccine 1

B) Response of mice to vaccine 2

(Two mice were culled due to poor condition)

Fig. 6.1 Immune Response of Mice to Vaccines 1 & 2 as determined by ELISA





6.1.7. Preparation of NS-0 for fusion

NS-0, a mouse plasmacytoma cell line was selected as a fusion partner for mouse spleen cells as this line does not make or secrete H or L chains. One week prior to fusion, 2 or 3 vials of NS-0 cells were taken from liquid nitrogen and thawed at 37°C and inoculated into 20 ml of RPMI 1640 supplemented with 10% FCS (section 2.7.2.). After 24h 30 ml of fresh medium was added. A log phase growth was maintained to be used in the fusion. On fusion day, adherent NS-0 were harvested by incubating for 10-15 min at room temperature with 10 mM EDTA in PBS (section 2.7.8.) washed twice in RPMI by centrifugation at 180 g for 5 min and resuspended to 10 ml in basic medium.

6.1.8. Fusion protocol

Fusion was performed according to the principle described by Kohler and Milstein (1975) except that polyethylene glycol (PEG) was the fusion agent.

1. NS-0 and spleen cell suspensions were counted in an improved Neubauer haemocytometer under uv light by mixing a 10 ul sample of each with an equal volume of acridine orange. The number of cells in each cell suspension was then calculated.

The following cell counts were obtained: spleen cells 1.1 x 10^8/ml. Two flasks of NS-0 contained A - 1.1 x 10^7/10 ml and B - 4.6 x 10^6/10 ml

2. Appropriate numbers of spleen cells and NS-0 (spleen cells: NS-0 ratio in A 5:1 and in B 12:1) were combined and centrifuged at 450 g for 5 min.

3. 1 ml of PEG (in 50% RPMI at pH 8.5, section 2.7.6.) at 37°C was added to the cell pellet over 2 min, swirling gently to resuspend cells and the suspension centrifuged at 140 g for 5 min.
4. Over 2 min, 5 ml basic medium was then added without disturbing the pellet. The cells were resuspended by swirling gently over another 3-4 min and centrifuged at 300 g for 5 min.
5. The supernate was poured off carefully and 5 ml 20% FCS in HAT (section 2.7.3.) added without disturbing the pellet. After leaving for 7 min, cells were resuspended and adjusted to a cell concentration of 2×10^6 cells/ml with 20% FCS in HAT.
6. Cell suspensions (100 ul containing 2×10^5 cells per well) were dispensed into the inner 60 wells of 96 well tissue culture plates which contained 100 ul of 1 in 3 mixed thymocyte medium (MTM section 2.7.5.) in 20% FCS/HAT medium as the feeder layer. The plates were placed in a box and incubated with humidity and 5% CO_2 at 37°C. The wells were examined for hybrids on day 7 after fusion and fed with 100 µl of 20% FCS/HAT. Supernates (100 µl) of 97 hybrids which were at least 10% confluent were removed and screened against whole cells by ELISA for specific antibody production.

Supernates of 20 hybrids gave optical densities of greater than 0.5 (see appendix 1.2). These hybrids were expanded from pots to flasks and the serum content of medium was reduced gradually from 20% to 15% to 10% (section 2.7.2.).

6.1.9. Cloning

Healthy growing hybrids were cloned by limiting dilution at 1 and 10 cells/well in 20% FCS containing 1 in 3 MTM. Clones were

tested for antibody production by testing the supernates against whole cells by ELISA. Only eight cell lines were found to be producing antibodies (appendix 1.3).

6.1.10. Freezing of cells

Several vials of cells from every line were frozen when they reached a sufficient density (10^6 - 10^7 cells per flask). Cells resuspended in freezing medium (section 2.7.7.) were frozen at -70°C overnight and then transferred to liquid nitrogen.

6.1.11. Production of ascites in mice

Adult Balb/c female mice were inoculated intraperitoneally with 0.5 ml Pristane (2, 6, 10, 14- tetramethylpentadecane - Sigma). Three days later, 10^7 cells from the required cell line were inoculated intraperitoneally into these mice. Ascitic fluid was collected by inserting a needle (23 gauge) into the peritoneum allowing fluid to drain into a sterile bijoux bottle. The fluid was clarified by centrifugation and stored at -20°C until used.

6.2. Characterization of monoclonal antibodies

6.2.1. Class and subclass determination

Determination of class and subclass of the culture supernates was performed using the mouse monoclonal antibody isotyping kit (Amersham International plc, Amersham, UK) according to the manufacturer's instructions. Table 6.1 gives the isotypes of the antibodies from the culture supernates.

Table 6.1. Class and subclass of monoclonal antibodies

Cell line	Class/subclass
1/2-16-8	IgM
1/4-1-6	IgG_1
1/8-16-11	IgG_3
1/13-6-12	IgG_{2b}
1/15-9-1	IgM
1/18-13-5	IgM
1/19-12-13	IgG_1
1/20-10-15	IgM

6.2.2. Western blotting

Western blots of whole cell lysates, OMP and LPS (proteinase k digests) of W674 bacteria grown in NB containing 2,2'-dipyridyl were prepared by the methods described in sections 2.6.3. and 2.6.4. The NC strips were then probed with culture supernates. Fig. 6.3, 6.4 and 6.5 demonstrate the results of whole cells, OMP and LPS blots respectively.

The antibodies secreted by 1/4-1-6 cells were directed against the 77 kDa protein, while antibodies of 1/8-16-11 were found to react with the LPS. The remaining six did not react with proteins or against LPS in Western blotting.

6.2.3. Indirect Haemagglutination Test (IHA)

Each mAb (ascitic fluid) was tested by the method described in section 2.6.1. The antigen was prepared from strain W674, the homologous immunizing strain. The test was performed with a series of doubling dilutions from 1 in 2 to 1 in 128 of each mAb. Fig. 6.6. shows the haemagglutination ability of each mAb. Only one mAb - 1/2-16-8 demonstrated haemagglutination to a titre of 1 in 64 and was considered to be reactive against a component of the capsule, as it did not react with LPS in the Western blot.

The specificity of the anti-capsule mAb was studied by testing it against fifteen different strains of *P. multocida* in IHA. The crude capsular extract of 15 strains was prepared by the method in section 2.3.2. The test was performed according to the method given in section 2.6.1. using 1 in 4 dilution of the mAb. The reactivity of the mAb against each strain was compared with that of

Fig. 6.3. Western blots of W674 whole cell lysate probed with neat monoclonal antibody culture supernates: a. 1/2-16-8; b. 1/4-1-5; c. 1/4-1-6; d. 1/8-16/11; e. 1/13-6-12; f. 1/15-9-1; g. 1/18-13-5; h. 1/19-12-13; i. 1/20-15-15; j. standard positive serum (from the mouse used for fusion); k. standard negative (culture medium 10% FCS).

Fig. 6.4. Western blots of outer membrane proteins of W674 probed with neat monoclonal antibody culture supernates. a. 1/8; b. 1/8-15; c. 1/8-16-11; d. 1/18-5; e. 1/18-13-5; f. 1/20-16; g. 1/20-10-15; h. 1/19-11; i. 1/19-12-13; j. 1/13-6 ; k. 1/13-6-12; l. 1/2-15; m. 1/2-16-8; n. 1/4-1-6; o. 1/15-9-1; p. standard negative (culture medium 10% FCS); q. standard positive serum (from mouse used for fusion)

Fig. 6.3.

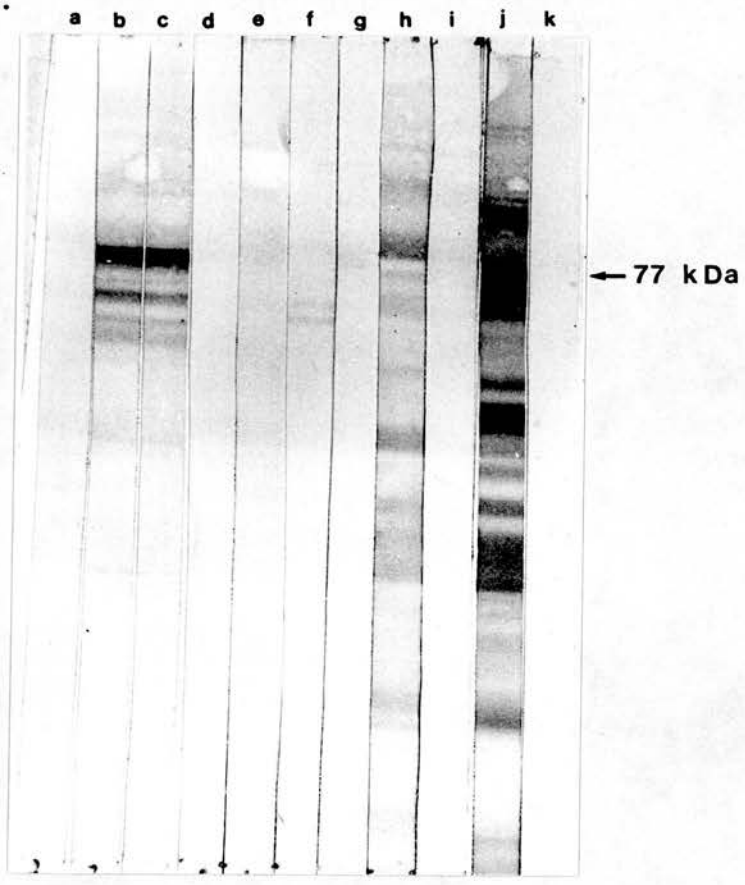


Fig. 6.4.

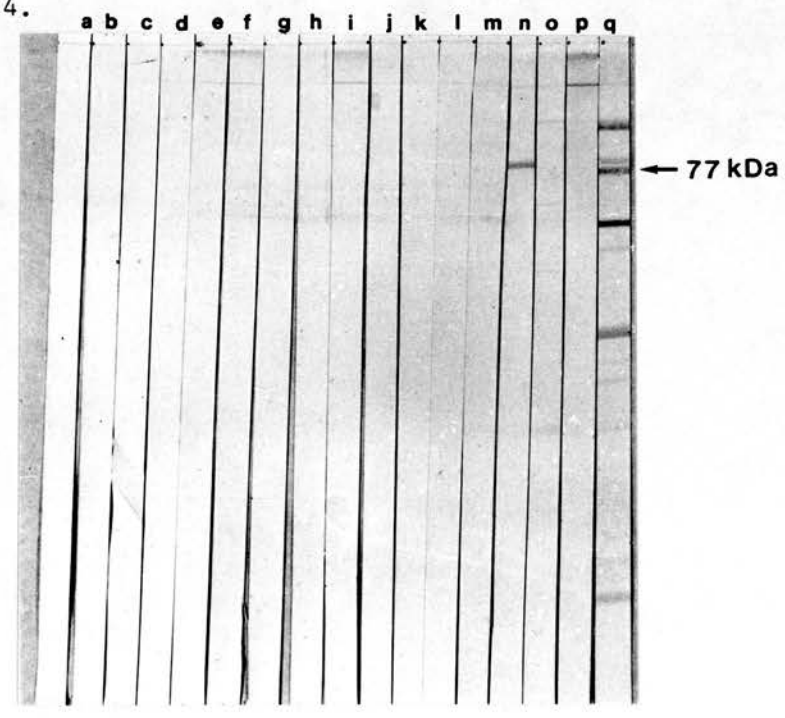


Fig. 6.5. Western blots of LPS (proteinase k digests) of W674 probed with neat monoclonal antibody culture supernates. a. 1/2-16-8; b. 1/4-1-6; c. 1/8; d. 1/8-15; e. 1/8-16-11; f. 1/13-6-12; g. 1/15-9-1; h. 1/15-9-6; i. 1/18-13-5; j. 1/19-11; k. 1/19-12-13; l. 1/20-10-15; m. standard negative (culture medium 10% FCS); n. standard positive serum (from the mouse used for fusion)

Fig. 6.5.

hyperimmune rabbit serum diluted 1 in 500 in PBS. Table 6.2 indicates the strains tested and summarizes the results. The type of haemagglutination demonstrated by both mAb and hyperimmune rabbit serum are shown in fig. 6.7.

It was evident that the haemagglutinating ability of the anti-capsule mAb was distinctly different from that of the polyclonal serum. The mAb demonstrated agglutination only with the homologous strain. A slight agglutination was observed with reference types A (10322) and D (10325).

6.2.4. Bactericidal activity of monoclonal antibodies as determined by in vitro assay

The bactericidal capacity of each mAb was tested by the method described in section 5.2.1. using the ascitic fluid. Sera from gnotobiotic lambs were used as the source of complement (section 5.2 (d)). The bacterial strain used was *P. multocida* W674, the homologous immunizing strain.

Complement-mediated bactericidal activity was demonstrated only by the anti-LPS mAb (1/8-16-11), which killed 80% of *P. multocida* organisms. The standard positive serum gave 100% killing while no bactericidal activity was observed with standard negative serum (fig. 6.8).

6.2.5. Ability of mAbs to protect mice passively

The protective capacity of the mAbs was studied by the passive mouse protection test (PMPT) using Balb/c mice. Each mAb (0.2 ml) in the form of ascitic fluid was inoculated intra-peritoneally

Table 6.2. Haemagglutination property of the anti-capsule mAb against fifteen different strains of *P. multocida* in comparison to the polyclonal hyperimmune serum.

Strain of *P. multocida* tested	Type of agglutination demonstrated by	
	mAb	polyclonal serum
Type A	±	-
Type B	-	-
Type D	±	-
Type E	-	-
X12 (bov. A)	-	+
X1053 (bov. A)	-	+
W666 (bov. A)	-	-
X109 (bov. A)	-	-
W829 (bov. A)	-	+
X110 (bov. A)	-	+
X1016 (bov. A)	-	+
X200 (bov. A)	-	+
X9 (bov. A)	-	+
W669 (bov. A)	-	+
W674 (bov. A. homologous)	+	+

\+ - agglutination
± - very slight agglutination
\- - no agglutination
bov. A - bovine type A

Fig. 6.6. The ability of monoclonal antibodies to agglutinate sheep red blood cells coated with heat-extracted capsular antigen (indirect haemagglutination). Row a, 1/2-16-8; b. 1/4-1-5; c. 1/8-16-11; d. 1/13-6-12; e. 1/15-9-1; f. 1/18-13-5; g. 1/19-12-13; h. 1/20-10-15; i. antigen coated red blood cells only (no monoclonal antibodies)

Fig. 6.7. Indirect haemagglutination of heterologous strains of *P. multocida* by (a) the anti-capsule monoclonal antibody (ascitic fluid) and (b) the polyclonal hyperimmune rabbit serum.

① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨ ⑩ ⑪ ⑫

⑬ ⑭ ⑮ ⑯

1. Type A (10322)
2. Type B (10323)
3. Type D (10325)
4. Type E (10326)
5. X12
6. X1053
7. W666
8. X109
9. W829
10. X110
11. X1016
12. X200
13. X9
14. W669
15. W674
16. antigen-coated red blood cells only

Fig. 6.6.

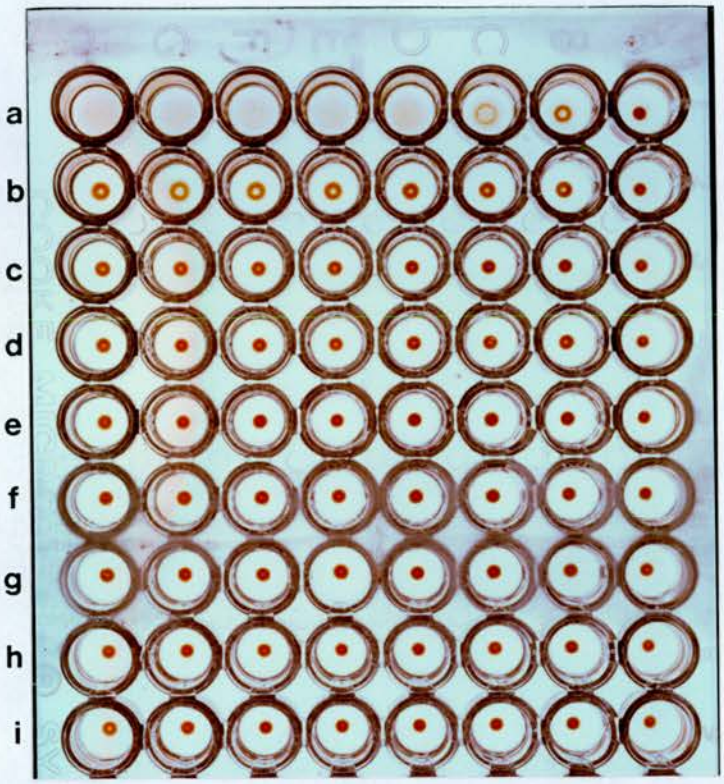
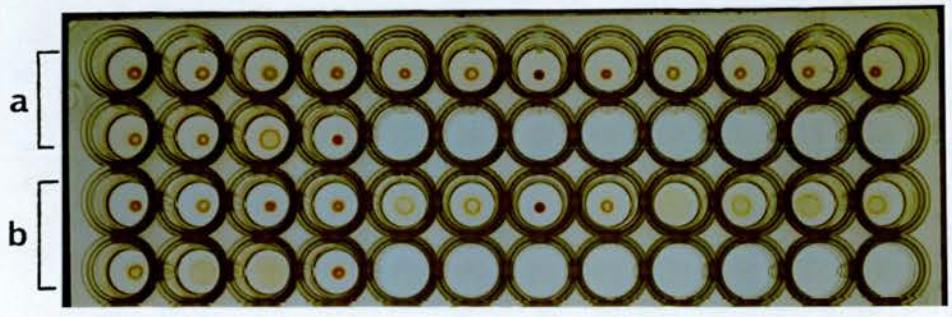


Fig. 6.7.



into five mice at 0h. Another group of five mice, used as the controls, received 0.2 ml (ascitic fluid) of a mAb - E_7 raised against louping-ill virus. One hour later, all mice were challenged intra-peritoneally with 0.2 ml of the bacterial suspension containing 2.0 x 10^8 viable *P. multocida*. This challenge inoculum consisted of approximately 10 LD_{50}. Mice were observed for up to 48h after challenge, deaths were recorded and the percentage protected was calculated. Table 6.3 indicates the protective capacity of mAbs as determined by the PMPT.

Of the mice surviving after challenge, only those which received anti-LPS mAb were found to be healthy. As the other mice showed severe illness, they were killed by cervical dislocation prior to the completion of the observation period.

Lack of growth of *P. multocida* in the heart blood plated onto SBA, of one of the 5 mice which received anti-LPS mAb and protected against challenge, confirmed the complete clearance of bacteria from the mouse circulatory system. In contrast, *P. multocida* could be re-isolated from heart blood of mice which received 1/19-12-13, 1/20-10-15 and E_7 (controls) mAb (fig. 6.9).

6.2.6. Ability of mAbs to enhance phagocytosis

By employing the methods described in sections 5.5.1 - 5.5.5 the ability of the anti-capsule and anti-LPS mAb to enhance phagocytosis by mouse peritoneal macrophages was studied. Heat-inactivated ascitic fluid of these mAbs were used in this assay. Decapsulation of *P. multocida* by incubating bacteria with hyaluronidase seemed to have no effect on this property of

Table 6.3. Ability of monoclonal antibodies to protect mice as determined by passive mouse protection test.

mAb	No. of mice survived out of five, after challenge	% protected
1/2-16-8 (anti-capsule)	0	0
1/4-1-6 (anti-77kDa protein)	0	0
1/8-16-11 (anti-LPS)	5	100
1/13-6-12	0	0
1/15-9-1	0	0
1/18-13-5	0	0
1/19-12-13	2	40
1/20-10-15	5	100
E_7 (control)	0	0

Fig. 6.8. Bactericidal activity (%K) of monoclonal antibodies as determined by *in vitro* assay

	a	b	c
1.	1/2-16-8	1/15-9-1	polyclonal immune mouse serum
2.	1/4-1-6	1/18-13-5	polyclonal hyperimmune rabbit serum (standard positive)
3.	1/8-16-11	1/19-12-13	normal mouse serum
4.	1/13-6-12	1/20-10-15	normal rabbit serum

Fig. 6.9. Evidence of complete clearance of bacteria from the mouse circulatory system by the anti-LPS monoclonal antibody.

a. Recovery of *P. multocida* from heart blood of mouse passively immunized with the monoclonal antibody 1/20-10-15

b. Recovery of *P. multocida* from heart blood of mouse passively immunized with the monoclonal antibody 1/19-12-13

c. Recovery of *P. multocida* from heart blood of control mouse

d. Lack of growth of *P. multocida* in the heart blood of mouse passively immunized with the monoclonal anti-LPS antibody 1/8-16-11.

Fig. 6.8.

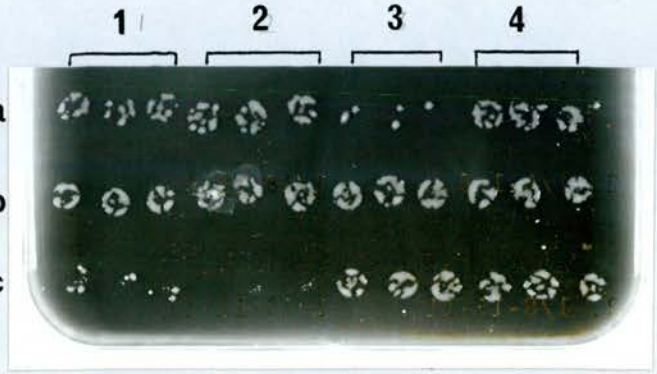
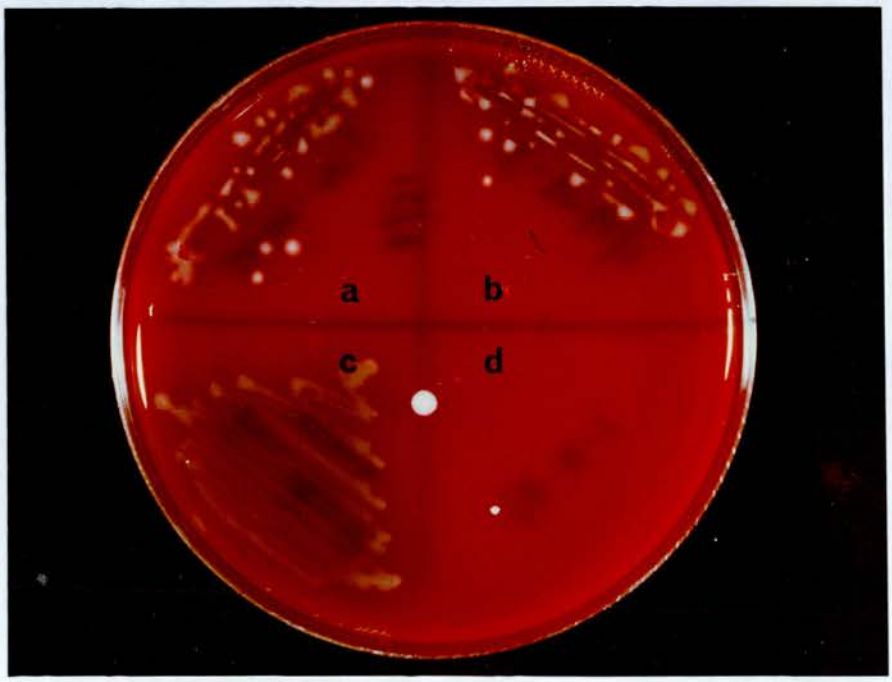


Fig. 6.9.



phagocytosis. The percentage phagocytosis exhibited by the anti-LPS mAb in the assay was greater than that of anti-capsule antibodies. Table 6.4 indicates the percentage phagocytosis of *P. multocida* by anti-capsule and anti-LPS mAb and standard mouse serum.

The two mAbs (1/19-12-13 and 1/20-10-15) that showed some degree of protection in the PMPT were not included in the phagocytosis assay as they had not been characterized.

6.2.7. Crossed immunoelectrophoresis of capsular components by mAbs

The crossed immunoelectrophoresis method described by Hancock and Poxton (1988) with modifications as to the voltage employed, was followed.

The antigen used in this study was the crude capsular extract described in section 2.3.2.

The first dimension gel was prepared by pouring 15 ml molten agarose onto a 80 mm square glass plate and allowing it to set. With the aid of a template (fig. 6.10a), wells were cut out and filled with 15 µl of the antigen. Bromophenol blue (BDH) was filled into the well at one of the corners and connected the edges of the gel to the buffer reservoirs with presoaked thick filter paper. First dimension electrophoresis was performed until the bromophenol blue reached a length of 9.0 mm in 45 min applying a voltage of 10 v/cm. The plate was taken out and strips were cut out. The strips were then carefully transferred to the edge of 50 mm square sheets of Gelbond (FMC Corp, Maine, USA) on a glass

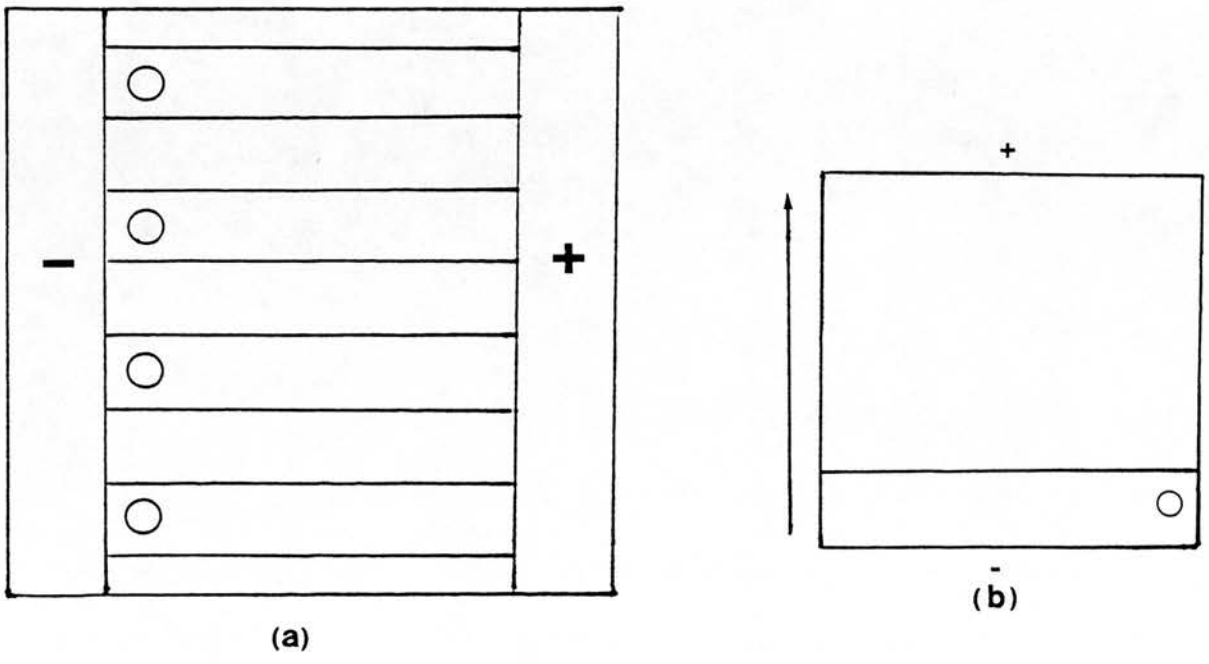
Table 6.4. The percentage of *P. multocida* W674 cells phagocytosed after opsonisation with anti-capsule and anti-LPS mAb

mAb or serum tested	% phagocytosis with no hyaluronidase treatment	% phagocytosis with hyaluronidase treatment
Anti-capsule (1/2-16-8)	9.9	8.9
Anti-LPS (1/8-16-11)	13.6	12.2
Standard negative (normal mouse serum)	0	0.5
Standard positive (chamber implanted mouse serum)	5.3	6.9

plate as shown in Fig. 6.10 (b). The second dimension gels were prepared by adding 300 ul of ascitic fluid of 1/15-9-1, 1/18-13-5 and 1/2-16-8 mAbs and polyclonal hyperimmune rabbit serum into each of 3 ml molten agarose mixing gently and casting this against the first dimension strip. Gels were arranged in the tank with the first dimension strips nearer to the cathode. Wicks were connected to the reservoirs as above and a voltage of 4 v/cm was applied overnight.

Fig. 6.10. Templates for crossed immunoelectrophoresis

(a) first dimension (b) second dimension



The gels were dried according to the method described by Hancock and Poxton (1988). When the gels were completely dried, they were stained for 10 min in Coomassie blue. The backgrounds were cleared in several changes of the destainer.

The anti-capsule mAb (1/2-16-8) produced a single precipitating peak while the polyclonal serum produced at least 7 peaks (Fig. 6.11 a & b). This confirmed the heterogeneity of the capsular extract and the polyclonal serum. The reactivity of the anti-capsule mAb seems to be directed against a major antigenic determinant of the capsule. None of the other mAb possessed the property of precipitation.

Part II. Further studies on the anti-LPS mAb

Results of preliminary work carried out on the mAbs revealed that antibodies directed against LPS mediated complement-mediated killing of homologous *P. multocida* and protected mice against a homologous challenge. As it was the only mAb which protected, it was decided to study both the strain specificity and structural specificity of the anti-LPS mAb.

6.3. Comparison of reactivity of anti-LPS mAb against different strains of *P. multocida* by indirect ELISA, Western blotting and bactericidal assay

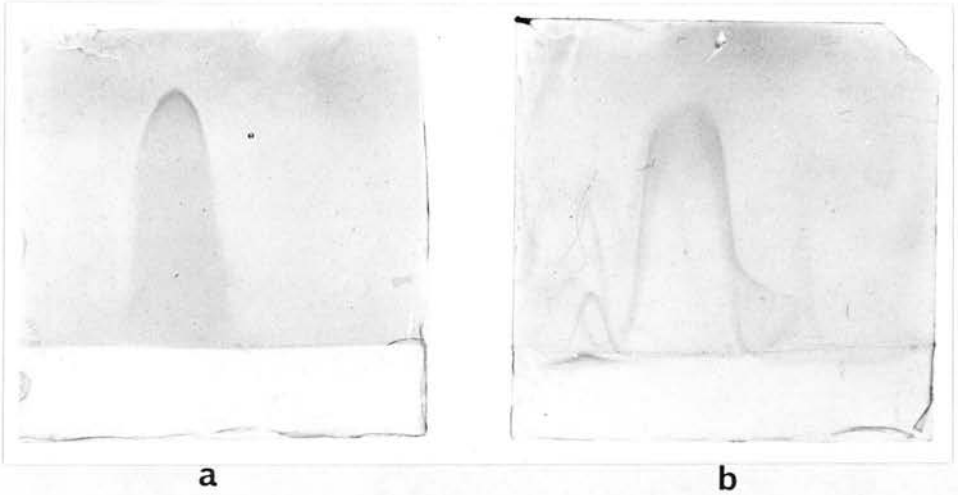
6.3.1. Screening of *P. multocida* and other gram-negative organisms by indirect ELISA

a) Bacterial strains

Fifty different strains of *P. multocida* randomly selected from

Fig. 6.11. Precipitation arcs produced by the anti-capsule monoclonal antibody 1/2-16-8 (a) and the polyclonal hyperimmune rabbit serum (b) in crossed immunoelectrophoresis. Heat extracted capsular-antigen (20 ul) run against 300 ul of either monoclonal antibody (ascitic fluid) or polyclonal hyperimmune rabbit serum

Fig. 6.11.



a b

the culture collection of Abdullahi (1987) including four type cultures of *P. multocida* serotypes A, B, D and E (Appendix 2.1) were tested against the anti-LPS mAb in a whole-cell ELISA. For comparison, all 16 serotypes of *P. haemolytica*, seven other Gram-negative bacteria (Appendix 2.1) and a Gram-positive *Listeria monocytogenes* (4B) were also included in this study.

b) Preparation of antigens for ELISA

An overnight culture from each strain grown in nutrient broth (10 ml) was washed once in PBS and resuspended in 0.5% formal saline to an absorbance at 420 nm of 0.1. This corresponded approximately to 10^7 cfu/ml. Wells of microtitre plates were coated with 100 ul of each bacterial suspension by incubating overnight at $4^{\circ}C$. The assay was then continued as described in section 2.6.2 with anti-LPS mAb culture supernatant as the primary antibody. Absorbance at 492 nm was read for each strain and the percentage absorbance in relation to the homologous strain (W674) was calculated. Fig. 6.12 shows the reactivity of the anti-LPS mAb against *P. multocida* and other Gram-negative bacteria determined by indirect ELISA.

The anti-LPS mAb recognized its epitope in LPS of 58% of the *P. multocida* strains tested (those above 50% absorbance). This epitope does not appear to be present in the LPS of the other Gram-negative bacteria tested. It also did not cross-react with any of the type strains of the four serotypes of *P. multocida*.

Fig. 6.12. Reactivity of anti-LPS monoclonal antibody against different strains of *P. multocida* and other Gram-negative bacteria as determined by indirect ELISA. The percent absorbance (OD) for each strain in relation to the homologous immunizing strain W674* is indicated.

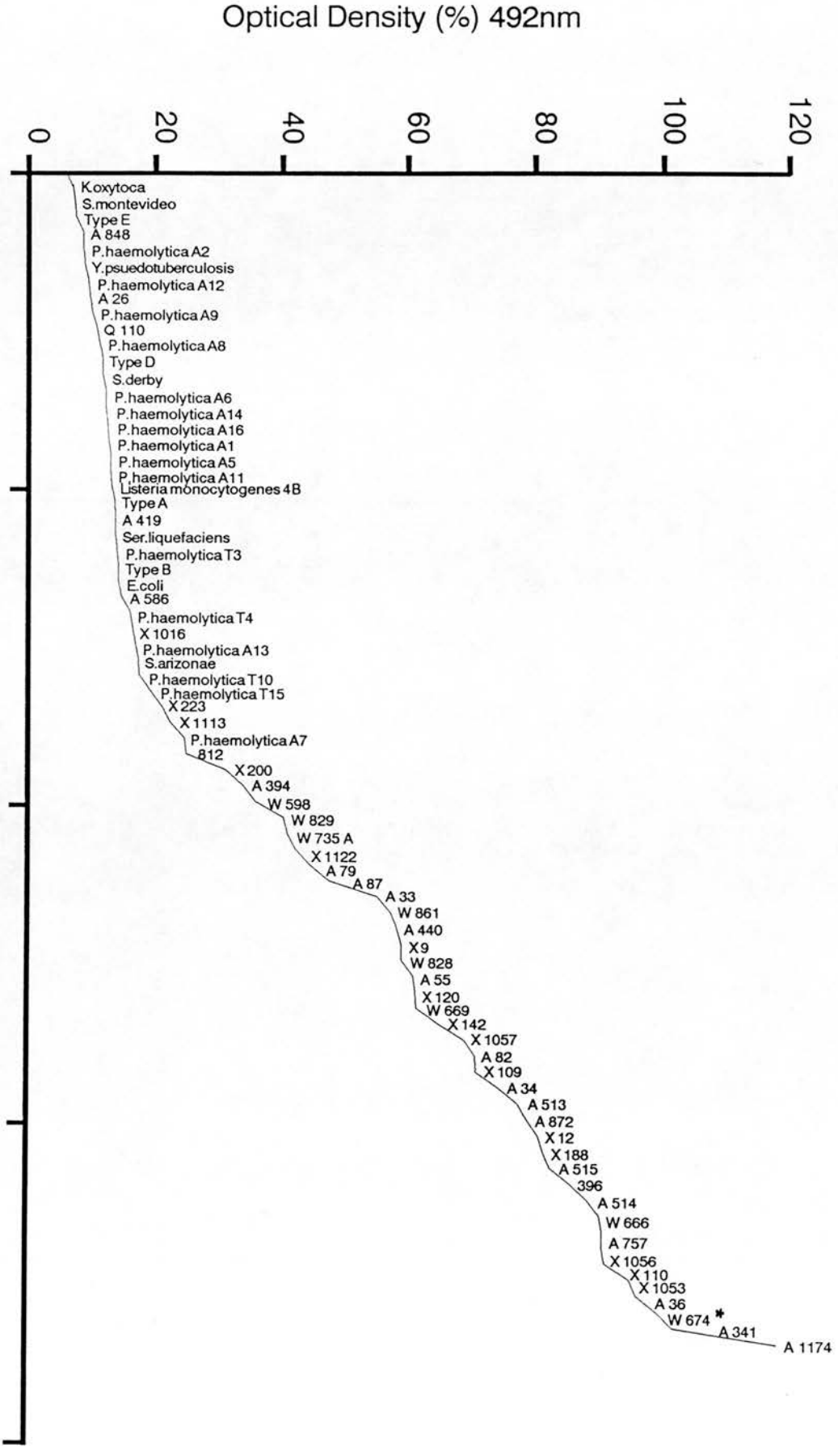


Fig. 6.12 Reactivity of Anti-LPS monoclonal antibody against Gram-negative bacteria determined by Indirect ELISA

c) Selection of strains for Western blotting and for bactericidal assay

A total of 20 isolates were selected based on the ELISA results, to be tested by Western blotting and by bactericidal assay. Of these, 10 strains including the homologous strain gave a percent absorbance of greater than 60 while the percentage absorbance indicated by the remaining 10 strains was less than 18.

6.3.2. Reactivity of the anti-LPS mAb against LPS of different strains of *P. multocida* by Western blotting

Proteinase K digests of twenty strains were separated on 14% polyacrylamide gels (without SDS) and transferred electrophoretically onto NC and probed with anti-LPS mAb culture supernate as described in section 2.6.4. The same antigens separated under identical conditions were stained with silver (Hancock and Poxton 1988) in order to study the pattern of LPS of each strain.

All the ten strains which gave a percent absorbance of greater than 60 in ELISA were recognized by the anti-LPS mAb in Western blotting. The mAb did not react with the LPS of strains that gave low % absorbance in whole-cell ELISA (figs. 6.13 a and b). The pattern of LPS of these strains resembled those of semi-rough strains of enterobacteria (figs. 6.13 c and d).

6.3.3. Bactericidal activity of the anti-LPS mAb against heterologous strains of *P. multocida*

This was performed essentially by the method described in

section 5.2.1. using ascitic fluid and sera from gnotobiotic lambs as the sources of specific antibody and the complement respectively. From each bacterial suspension several dilutions were included in the test, in order to arrive at the optimum assay count which should ideally be 1.0×10^3 cfu/ml. Each strain was tested against hyperimmune rabbit serum for comparison. Table 6.5 summarizes the results of bactericidal activity of mAb against different strains of *P. multocida*. Complement-mediated killing by the anti-LPS mAb was observed in ten of the twenty strains of *P. multocida* tested. These strains also gave high percent absorbances in whole-cell ELISA and were recognized by the mAb in Western blotting. No bactericidal activity was demonstrated by the mAb against the remaining ten strains tested.

Correlations of % killing and % absorbance were determined by the Spearman-Jackson rank correlation test and were found to be highly significant ($P < 0.001$).

6.4. Study of the anti-LPS mAb for its heterologous protection by passive protection in mice

The protective capacity of the anti-LPS mAb against heterologous strains of *P. multocida* was assayed by the method described in section 6. For this study, six strains were selected based on results of the reactivity of the mAb against different strains in ELISA and Western blotting. Five of these strains reacted well with the mAb in both tests, while the remaining strain (A848) did not react with the mAb in either of these tests. The strains were A36, X110, X1053, A341, A1174 and A848.

Fig. 6.13. a. and b. Western blots of LPS (proteinase k digests) of heterologous strains of *P. multocida* probed with the anti-LPS monoclonal antibody 1/8-16-11 culture supernate (1 in 2 dilution)

a. Lane 1, A757; 2, A36; 3, X110; 4, X1056; 5, A848; 6, W666; 7, Q110; 8, X1016; 9, W674; 10, X120

b. Lane 1, A341; 2, A1174; 3, A586; 4, A26; 5, A419; 6, X1053; 7, Type A (10322); 8, Type B (10323) 9, Type D (10325); 10, Type E (10326)

Fig. 6.13. (a)

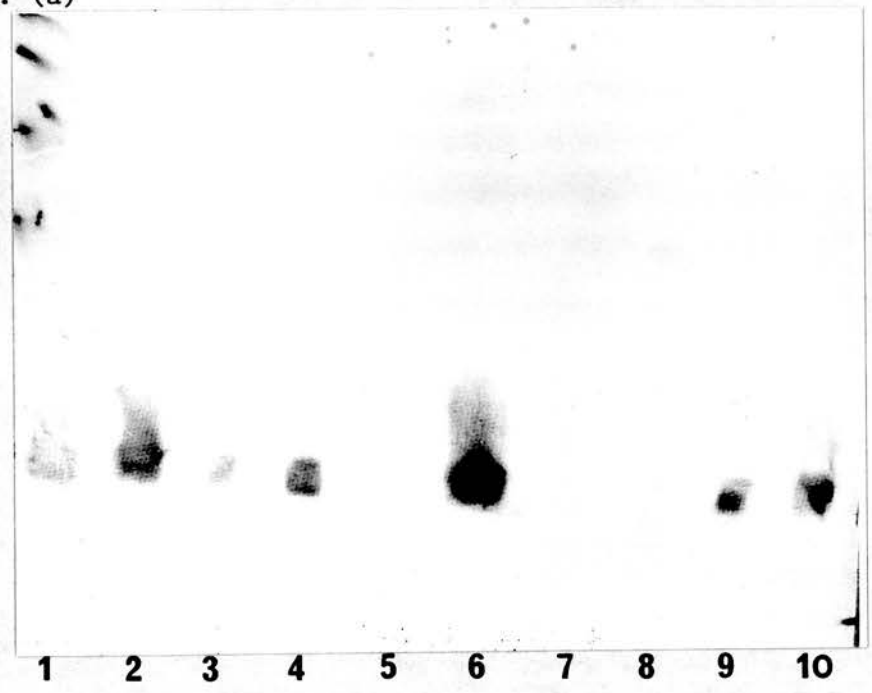


Fig. 6.13. (b)

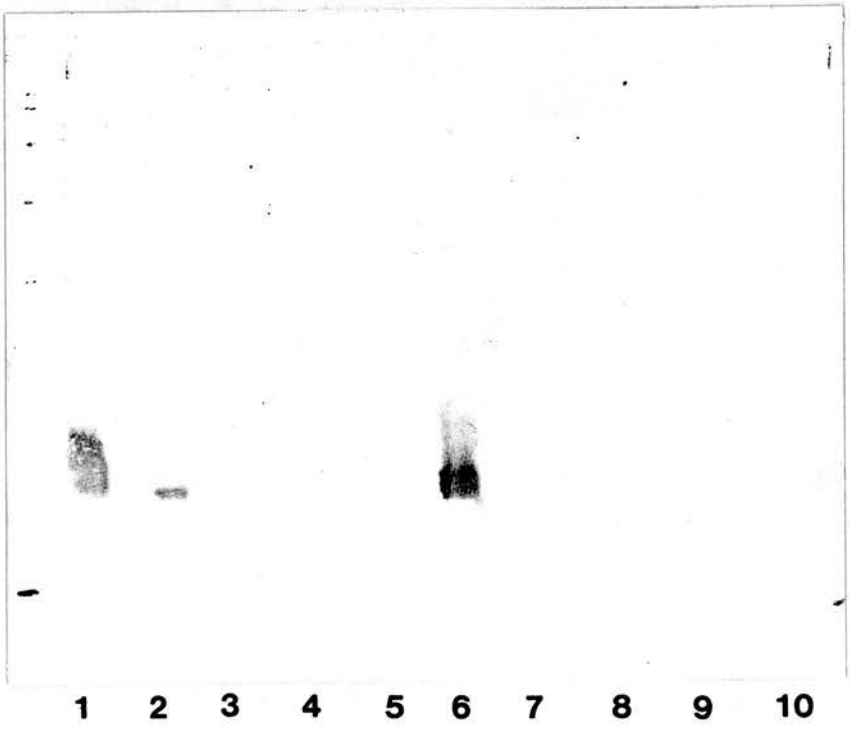


Fig. 6.13. c. and d. LPS (proteinase k digests) of strains of *P. multocida* used in fig. a. and b. stained with silver. c) Lane 1, A757; 2, A36; 3, X110; 4, X1056; 5, X1056; 6, W666; 7, Q110; 8, X1016; 9, W674; 10, X120; 11, A341; 12, A1174; 13, A586; 14, A26; 15, A419; 16, X1053

d) Lane 1, Type A (10322); 2, Type B (10323); 3, Type D (10325); 4, Type E (10326); 5, X110; 6, X1056; 7, A848

Fig. 6.13. (c)

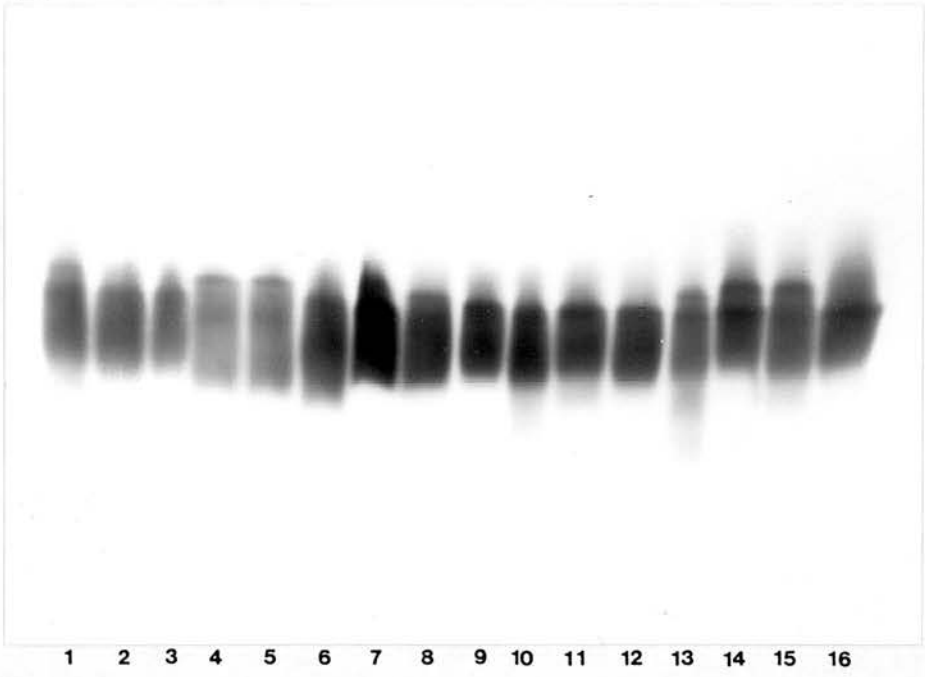


Fig. 6.13. (d)

Table 6.5. Bactericidal capacity of the anti-LPS mAb against different strains of *P. multocida* in comparison to the polyclonal serum and the corresponding ELISA percent absorbances of the same strains with mAb

Strain of *P. multocida* examined	% K by the mAb	% K by the polyclonal serum	% Absorbance determined by ELISA
X1056*	100	100	91.6
A36*	100	100	97.0
A1174*	100	100	119.2
A757*	71.7	83.4	91.6
X120*	100	100	62.0
X110*	78.8	95.5	92.0
W674*	95.8	100	100.0
W666*	100	100	91.2
X1053*	80	100	95.9
A341*	100	100	102.6
Q110	12	0	11.5
Type A (10322)	0	0	14.3
Type B (10323)	5	0	14.9
Type D (10325)	0	0	12.4
Type E (10326)	0	0	8.2
A848	0	0	9.3
X1016	0	0	17.1
A26	0	0	10.4
A419	0	0	14.4
A586	0	0	16.0

* strains of *P. multocida* which were recognised by the mAb in Western blotting

6.4.1. Determination of the LD_{50} of the six strains that were used in passive mouse protection test

The LD_{50}s for all six strains were studied by the procedure described in section 2.4.2. and are given in Table 6.6.

In the test proper, two groups of thirty Balb/c female mice were passively immunized at 0h. One group of mice received the anti-LPS mAb in the form of ascitic fluid while the other group received ascitic fluid of an unrelated mAb (E_7 - against louping-ill virus) which served as controls. One hour later 5 mice from both groups were challenged with a 10 LD_{50} of each strain.

Table 6.7 indicates the number of mice which survived and the percentage protected on challenge after administration of the mAb into mice. The control mice which received an unrelated mAb died after challenge with heterologous strains. The anti-LPS mAb was not protective against strain A848 which did not react in the serological assays discussed previously.

6.5. Determination of structural specificity of the anti-LPS mAb by the inhibition of ELISA

The inhibition of ELISA, which is a modification of indirect ELISA, according to Hancock and Poxton (1988) can be used to study the part played by purified antigen or components of antigen in a complex antigen/antibody reaction. In this technique (fig. 6.14) the plate is (a) coated with the complex antigen i.e. whole cells or cell envelope preparation. (b) Antiserum that has been pre-incubated with a purified component of the complex antigen e.g. LPS is then added. (c) Only antibodies that have not already bound

Table 6.6. The LD_{50} values of six strains which were used in PMPT

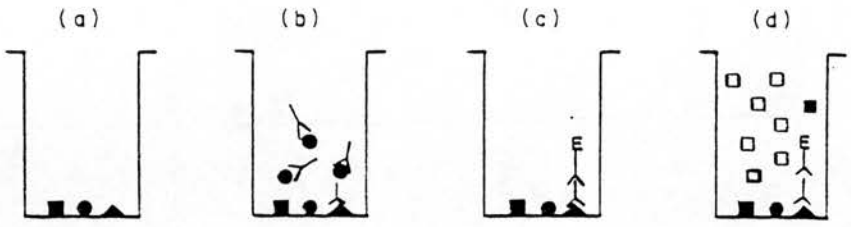
P. multocida strain	Viable count (cfu/ml) in the 6h broth culture	LD_{50} in terms of titre	LD_{50} in terms of No. of organisms
A36	1.5×10^9	$10^{-2.49}$	4.8×10^6
X110	1.2×10^9	$10^{-3.52}$	3.6×10^5
X1053	2.5×10^9	$10^{-2.45}$	8.8×10^6
A341	1.5×10^9	$10^{-3.37}$	6.3×10^5
A1174	1.0×10^9	$10^{-2.21}$	6.1×10^6
A848	8.5×10^8	$10^{-5.65}$	1.9×10^3

Table 6.7. Protective capacity of the anti-LPS mAb against heterologous strains as determined by PMPT

Challenge serum strain	No. of mice out of five which survived after challenge	% protected
A36	3	60
X110	3	60
X1053	3	60
A341	5	100
A1174	4	80
A848	0	0

to soluble antigen can bind to the immobilized antigen on the plate. (d) After substrate is added, the colour change is proportional to antibodies bound to this plate. A low optical density, therefore reflect the high proportion of antibodies present in the original antiserum to the specific antigen with which it is pre-incubated.

Fig. 6.14. Inhibition of ELISA



This technique was applied to study the specificity of the anti-LPS mAb using culture supernatant as the source of antibodies. The complex antigen selected to coat the plate was a cell envelope preparation. The inhibitors used were the cell envelope preparation, whole LPS and polysaccharide and lipid A fractions of the LPS molecule.

6.5.1. Preparation of antigens for the indirect ELISA

a) Cell envelopes

Cell envelopes were prepared from *P. multocida* W674 grown in NB containing 2,2'-dipyridyl by the method described under section 2.3.4, stopping the procedure at the stage of pelleting the cell

envelopes after sonication. The envelope pellet was then washed twice and resuspended in 3 ml of 0.1M Tris buffer at pH 7.4 and freeze-dried.

b) LPS

This was prepared essentially by the phenol water extraction method detailed in section 2.3.3. (a). Since the stability of LPS is dependent on its salt-form, the LPS was deionized by electrodialysis and converted to a uniform soluble salt-form as described by Hancock and Poxton (1988).

c) Deionization of LPS by electrodialysis

The apparatus developed by ISCO for the electrophoretic concentration of proteins was used for this purpose. The LPS (50 mg), reconstituted in 10 ml of pyrogen-free distilled water, was electrodialysed under the conditions stated by Hancock and Poxton (1988). The insoluble deionized LPS was made into a uniform suspension by placing it in a sonicating water bath for 5 min. The pH of the suspension was 3.6 and was at a concentration of 2 mg/ml. After neutralization with a small drop of 1M triethylamine, the LPS was made soluble and the pH was found to be 6.9.

d) Preparation of Lipid A

Deionized LPS at a concentration of 2 mg/ml in 1% acetic acid was heated at 100^{o}C for 90 min. The lipid A was precipitated from solution and was removed by centrifugation at 1000g for 10 min. The supernatant was saved for extraction of polysaccharides. The

precipitate was washed 3 times with hot distilled water and made soluble by neutralization with triethylamine and heating at 60°C. It was then stored freeze dried until required.

e) Preparation of polysaccharide

After removal of the lipid A precipitate from the acid hydrolysed solution, polysaccharide was extracted from the solution with an equal volume of chloroform-methanol (2:1 by volume). The aqueous phase consisting of the polysaccharide fraction was rotary evaporated to dryness.

The cell envelope preparation, LPS, lipid A and polysaccharide fractions were reconstituted to 1 mg/ml in PBS before being used in the assay. The optimum dilutions of the antigen (cell envelopes) and the antibody (mAb culture supernatant) were determined by a checker-board titration and found to be both 1 in 32.

6.5.2. The inhibition of ELISA

The wells of a microtitre plate were coated with the cell envelope preparation diluted 1 in 32 in carbonate/ bicarbonate buffer, pH 9.6, by incubating overnight at 4°C. The doubling dilutions of each inhibitor made in PBS were pre-incubated with equal volumes (200 µl) of 1 in 32 diluted mAb culture supernate for 30 min at 37°C. These pre-incubated samples, as well as the mAb culture supernate diluted 1 in 32, were added to the antigen coated wells after washing the wells. The assay was then continued as described in section 2.6.2.

The mean absorbance (OD) of the culture supernatant was taken from eight readings and was 0.920. The percent absorbance by the inhibitors at each dilution was then calculated. Fig. 6.15 demonstrates the pattern of inhibition indicated by the complex antigens and the fractions of LPS. The results show that the specificity of the anti-LPS mAb is directed towards the lipid A of the LPS molecule.

6.6. Structure of LPS of *P. multocida*

The study on determination of the structural specificity of the anti-LPS mAb by ELISA inhibition suggested that it was directed against the lipid A moiety of the LPS molecule. This anti-LPS monoclonal antibody reacted with 58% of *P. multocida* strains examined.

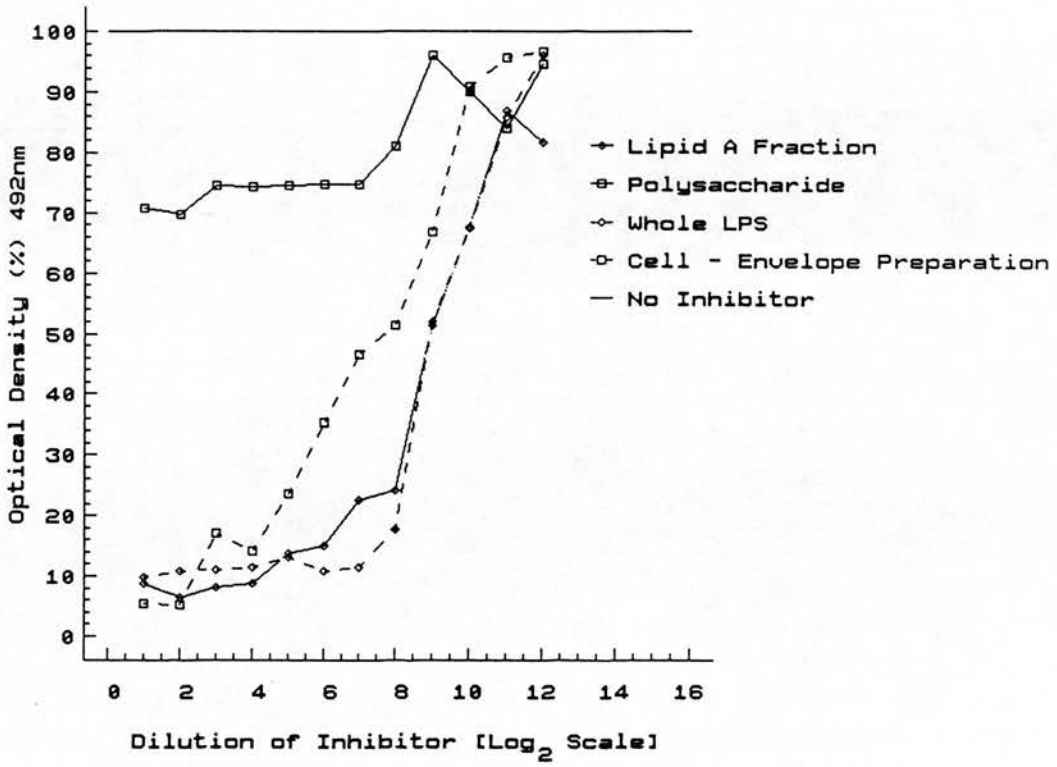
The structural differences of the LPS molecule in relation to the recognition and non-recognition of strains by the mAb constituted further investigations in which two strains were employed: strain W674 against which the mAb were produced and strain A26 with which the mAb did not react.

6.6.1. Preparation of LPS and fractionation of LPS into Lipid A and polysaccharide

The LPS of strain W674 and A26 was prepared from 4g dried bacteria of each strain by the procedure described in section 2.3.3. (a) and yielded 120 and 75 mg dry weight, respectively. One third of each preparation was used for the analysis of the whole molecule of LPS. Lipid A and polysaccharide fractions were

Fig. 6.15. Inhibition of ELISA to determine the structural specificity of this anti-LPS monoclonal antibody. The 100% value (—) was obtained for the mAb culture supernate by reacting it at a dilution of 1 in 32 it with the cell envelope antigen which was coated onto the plate. The inhibitors were lipid A fraction (-•-), polysaccharide fraction (-⊕-) of LPS, whole LPS (-○-) and cell-envelope preparation (-○-).

Fig. 6.15 Inhibition of indirect ELISA to determine the specificity of the anti-LPS monoclonal antibody



prepared from the remaining two thirds by mild acid hydrolysis, as described in sections 6.5.1. (d) and 6.5.1. (e). These two fractions and whole LPS were resuspended in pyrogen free distilled water at a concentration of 5-8 mg/ml.

6.6.2. Chemical analysis on whole LPS, lipid A and polysaccharide fractions

The chemical analysis on whole LPS, lipid A and polysaccharide fractions of the two strains for 3-deoxy-D-manno-octulosonic acid (KDO) carbohydrates and phosphates were carried out by thiobarbituric acid, phenol/sulphuric and sulphuric/perchloric acid method respectively as described by Hancock and Poxton (1988). Table 6.8 summarizes the results.

The LPS of both W674 and A26 strains were of similar chemical compositions (Table 6.8).

6.6.3. Separation and quantitation of sugars as their alditol acetates by gas chromatography

Lipid A was hydrolysed in 4M HCl for 6h at 100°C while the hydrolysis of polysaccharide was carried out in 2M HCl for 2h at 100°C. Alditol acetates were then prepared from hydrolysed lipid A and polysaccharide as described by Poxton and Cartmill (1982) and analysed by gas chromatography.

Sugars of the polysaccharide fraction of A26 and W674 in molar proportions in relation to heptose calculated from area percentages are given in Table 6.9.

The sugar compositions of the two strains are distinctly

Table 6.8. Composition of the whole LPS, lipid A and polysaccharide fraction (μg/mg LPS) of strains W674 and A26

Preparation	Composition in μg/mg LPS					
	W674			A26		
	KDO	Carbohydrate	Phosphate	KDO	Carbohydrate	Phosphate
LPS	92	220	3.0	91	238	3.0
Lipid A	4	27.4	1.2	1	42.6	0.9
polysaccharide	120	262.6	1.8	145	204.7	3.6

Table 6.9. Comparison of sugar composition of the polysaccharide fraction of A26 and W674 LPS

Sugar	Molar proportions of sugars in	
	A26	W674
Ribose	0.18	1.3 0.13
Galactose	0.75	0.4
Glucose	0.75	1.4
Unknown	0.27	0.28
Heptose	1.0	1.0
Glucosamine	0.18	0.1
Galactosamine	0.13	0

different from one another. The proportion of glucose was nearly 3 or 4 times that of galactose in strain W674 while equal proportions of glucose and galactose were observed in strain A26 (figs. 6.16 a and b). *P. multocida* strain A26 contained galactosamine, which was absent in strain W674. Both contained glucosamine (figs. 6.17 (a) and (b)).

6.6.4. Analysis of fatty acid composition of lipid A fractions of W674 and A26 as methyl esters by gas chromatography.

The fatty acids of lipid A of W674 and A26 were converted to methyl esters by the method of Poxton and Cartmill (1982) and analysed by gas chromatography.

The composition of fatty acids of lipid A fraction of the two strains are given in Table 6.10. The amounts indicated are in molar proportions in relation to 3-hydroxytetradecanoate (3 OH C14:0) calculated from area percentages. The molar proportions of fatty acids varied in that W674 contained a higher proportion of the tetradecanoate (C14:0) and less of the hexadecanoate (C16:0) as shown in Table 6.10.

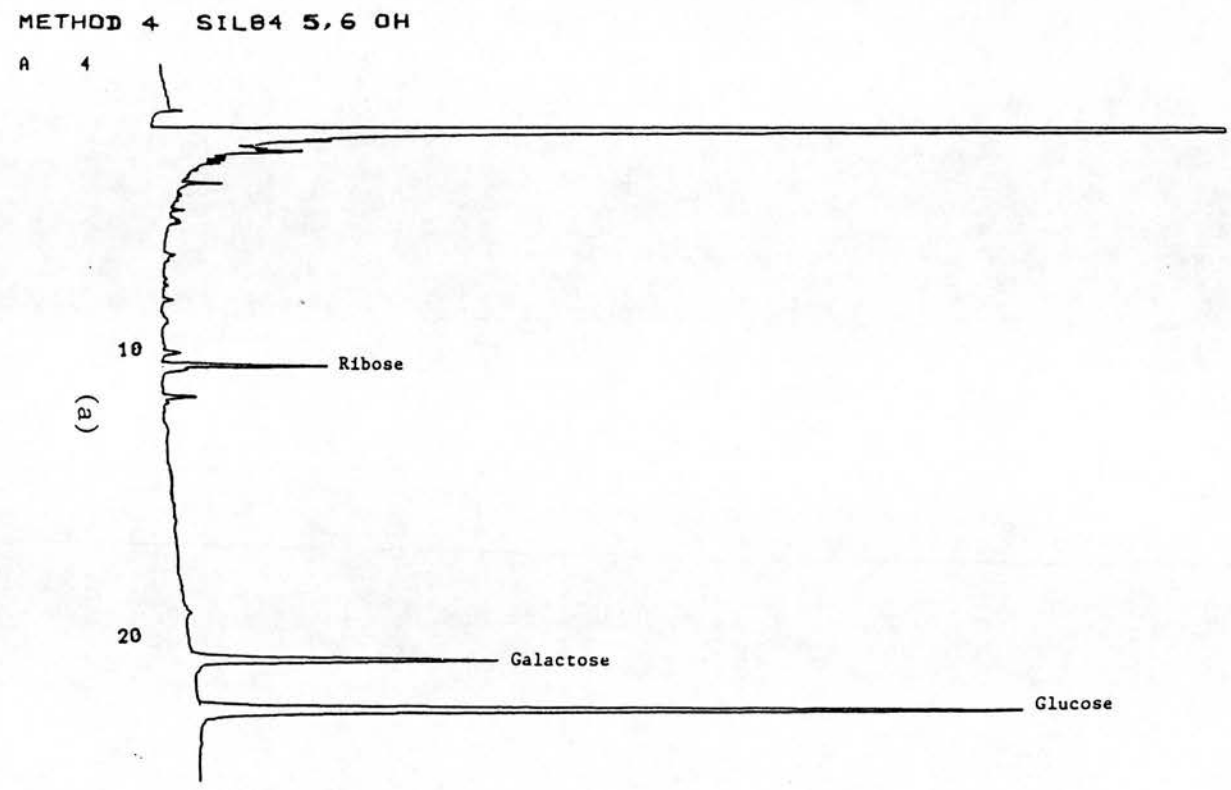
The chromatogram of lipid A of W674 is shown in fig. 6.18. Strain A26 also demonstrated a similar pattern and therefore not indicated here.

6.7. Fusion two

A second fusion was performed with mouse nos. 6 and 7 (fig. 6.1 (b)) after iv boost 3 days prior to fusion. A total of 153 hybrids were screened for antibody production and only 11 were

Fig. 6.16. Gas chromatograms of alditol acetate derivatives of hydrolysed polysaccharide fraction of LPS of W674 (a) and A26 (b) on a capillary column of SIL 84 for the detection of sugars. Temperature programme was 220°C for 2 min, 1°C/min to 240°C for 2 min

Fig. 6.16.


METHOD 4 SIL84 5,6 OH
A 4
10
Ribose
(a)
20
Galactose
Glucose

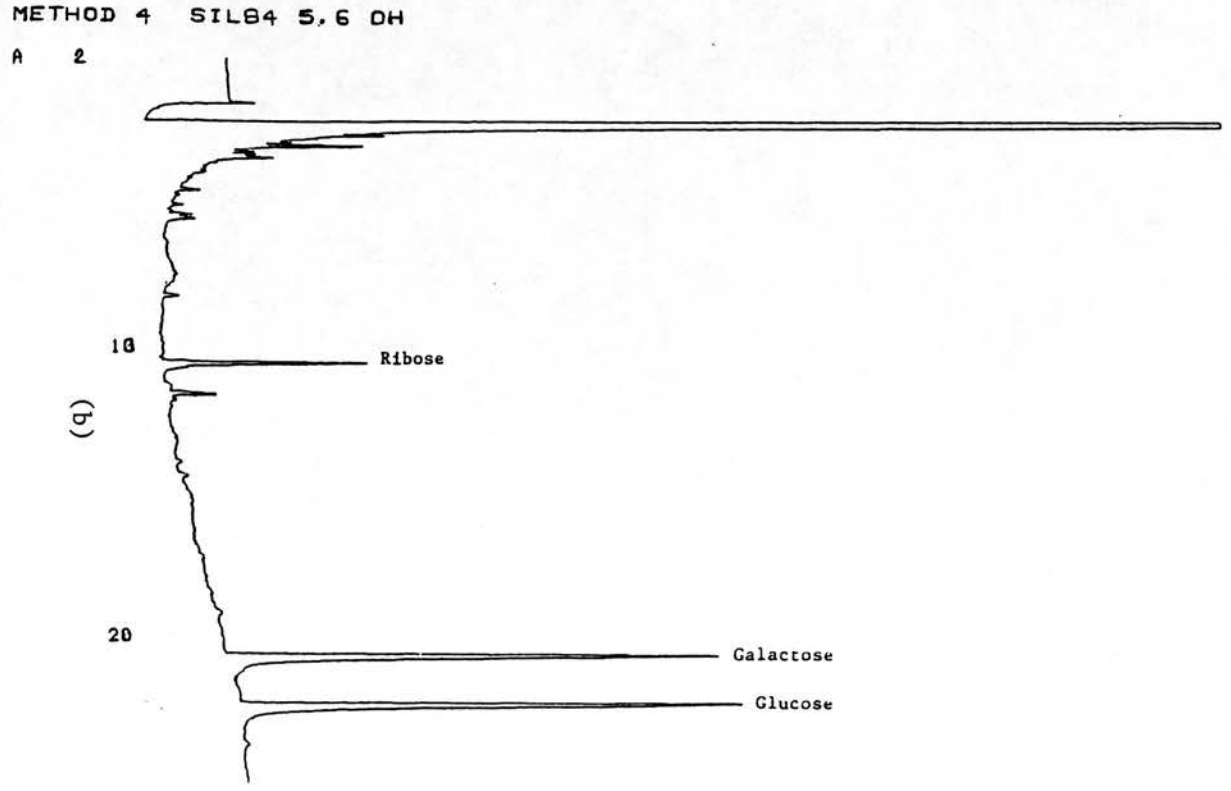
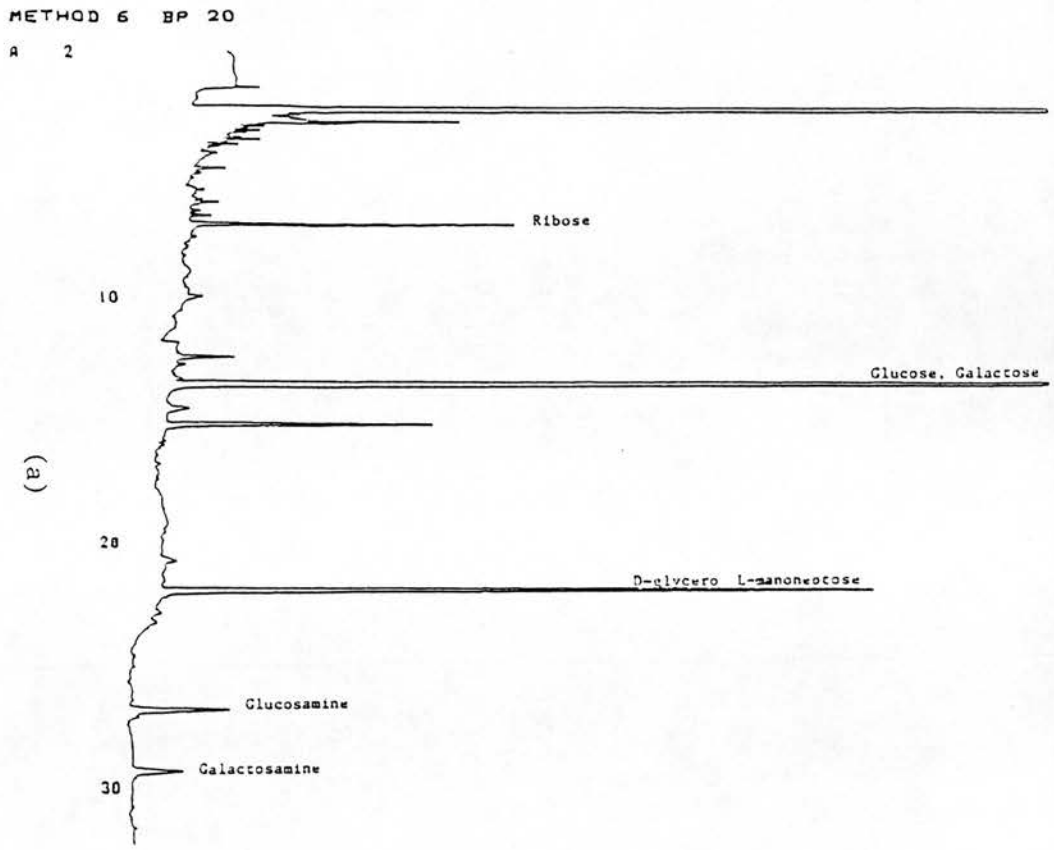

METHOD 4 SIL84 5,6 OH
A 2
10
Ribose
(b)
20
Galactose
Glucose

Fig. 6.17. Gas chromatograms of alditol acetate derivatives of hydrolysed polysaccharide fraction of LPS of A26 (a) and W674 (b) on a capillary column of BP 20 for the detection of sugars. Temperature programme was 220°C for 4 min, 2°C/min to 240°C for 4 min, 2°C/min to 250°C for 10 min.

Fig. 6.17.


METHOD 6 BP 20
A 2
Ribose
10
Glucose, Galactose
(a)
20
D-glycero L-manoheptose
Glucosamine
Galactosamine
30

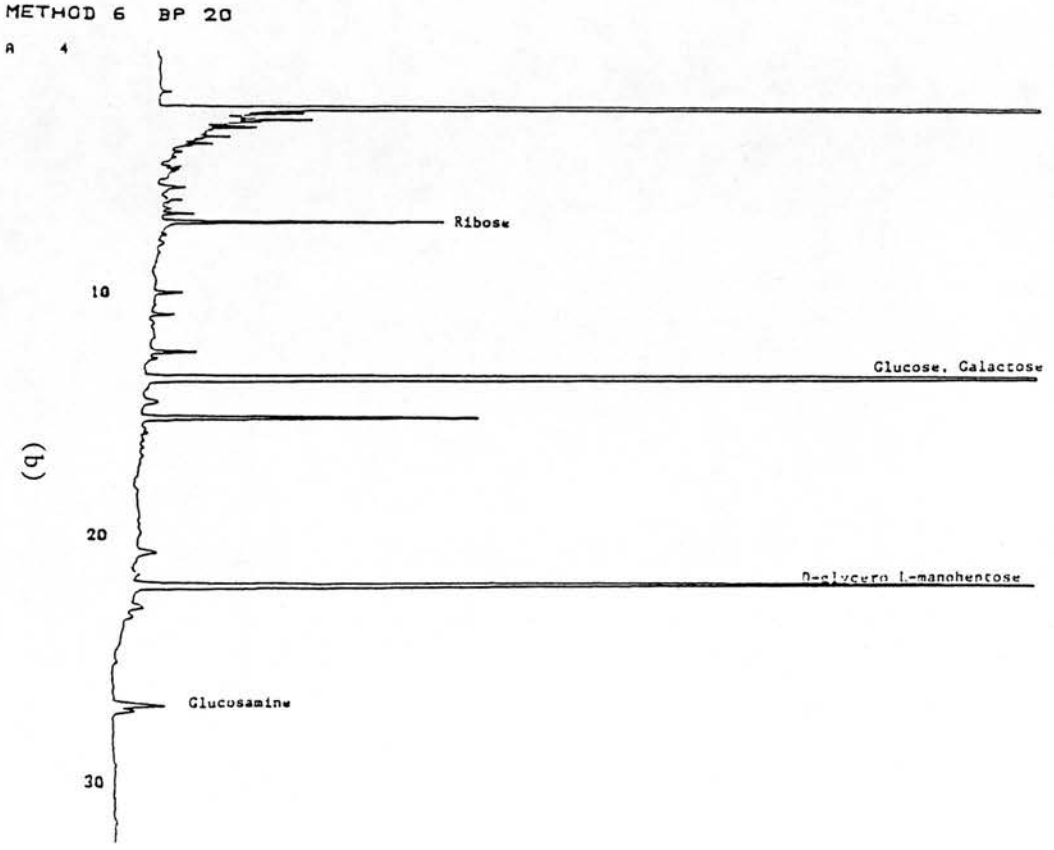

METHOD 6 BP 20
A 4
Ribose
10
Glucose, Galactose
(b)
20
D-glycero L-manoheptose
Glucosamine
30

Table 6.10. Comparison of the lipid A fraction of A26 and W674 LPS

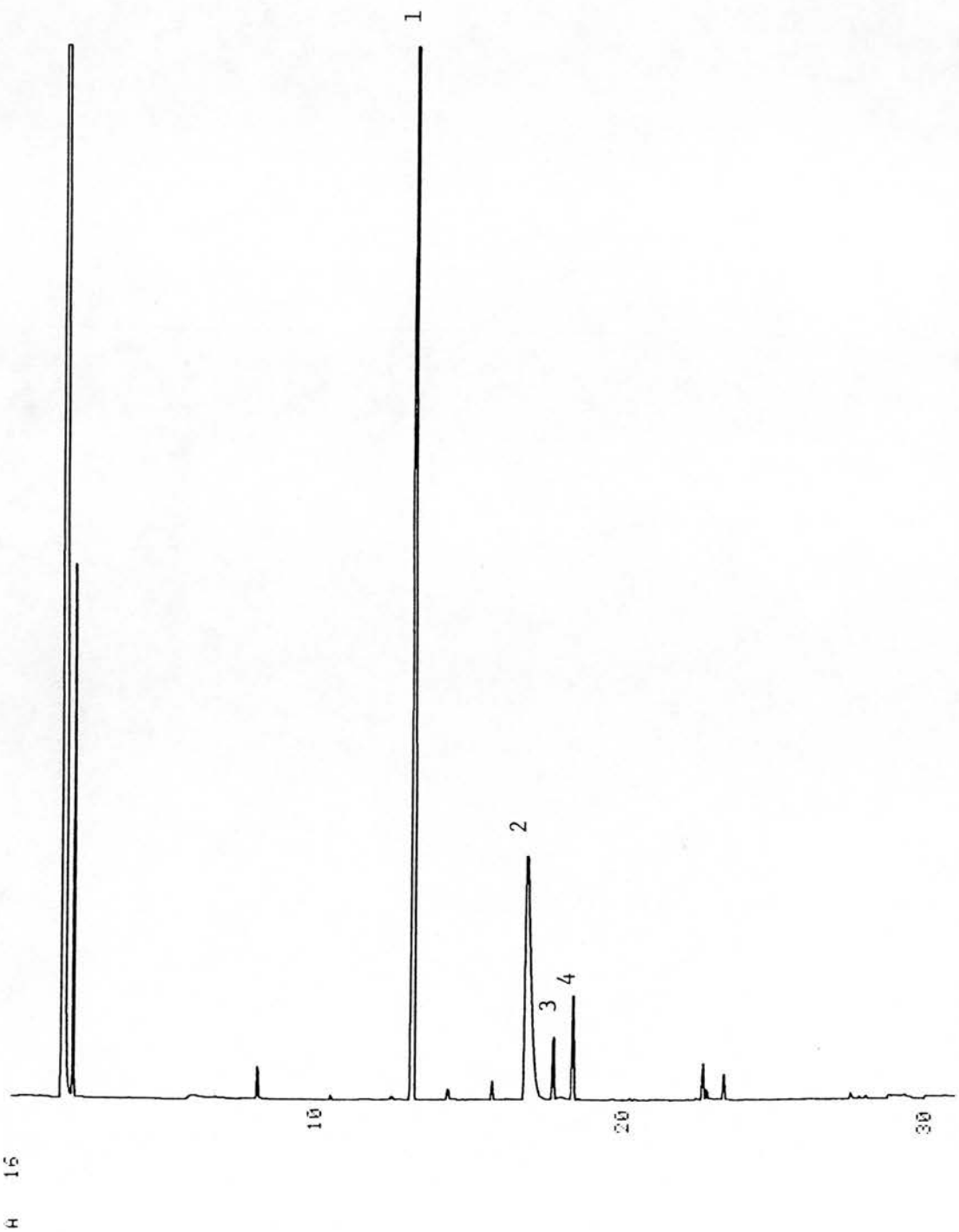
Fatty acid	Molar proportions of fatty acids in	
	A26	W674
Me. tetradecanoate (C14:0)	1.25	2.0
Me. 3-hydroxy-tetradecanoate (3OH C14:0)	1.0	1.0
Me. Cis-9-hexadecanoate ($C16:1^{9}$)	0.08	0.1
Me. hexadecanoate (C16:0)	0.25	0.1

Fig. 6.18. Gas chromatogram of methyl ester derivatives of methanolysed lipid A fraction of LPS of W674 on a capillary column BP 1 for the detection of fatty acids. Temperature programme was 150°C for 4 min, 4°C/min to 250°C for 2 min.

1. Me. tetradecanoate (C14:0)
2. Me. 3-hydroxytetradecanoate (3OH C14:0)
3. Me. Cis-9-hexadecanoate ($C16:1^{9}$)
4. Me. hexadecanoate (C16:0)

Strain A26 showed a similar fatty acid profile

Fig. 6.18.



found to be positive in whole-cell ELISA. Of these, three cell lines continued to produce antibodies after the second cloning by limited dilution method. An IgM antibody (2/4-7-9) was found to be reactive against LPS (proteinase K digests) in Western blotting. One other mAb (2/8-9-26) was positive in dot blots against the crude capsular extract and belonged to IgM class. The remaining mAb which was an IgG_3 did not react with proteins or against LPS in Western blotting. No further studies could be carried out on these mAb because of the time limit.

DISCUSSION

Monoclonal antibodies were raised by immunizing mice with *P. multocida* whole cells. Only three out of eight mAb could be characterized. These were specific for capsule, LPS and an outer membrane protein.

The dominant precipitation arc observed in crossed immunoelectrophoresis and the low IHA titre at which the antibodies agglutinated capsule-coated red cells suggested that the antibodies might be directed against the hyaluronic acid capsule. This notion is supported by the fact that the capsule of mucoid type A is largely composed of hyaluronic acid (Carter 1958) which is neither immunogenic nor specific (Carter and Annau 1953). Although this antibody agglutinated capsule-coated red cells with the homologous strain only, a slight agglutination was observed with serotypes A and D type cultures which suggested that the antigen was present in both of these strains but was lacking in all the other strains of type A. The presence of hyaluronic acid in most type A strains and

to a lesser extent in type D strains was reported by Carter (1972b). The haemagglutination pattern observed in this study (table 6.2) with different strains, and polyclonal rabbit serum indicates the specificity of this test. Not all type A strains, however, were agglutinated with rabbit antiserum. The involvement of LPS in this assay has been indicated by Bain and Knox (1961) and Carter and Rappay (1963) bringing into question the serotype specificity of this assay.

The hybridoma cells which produced the mAb (1/2-16-8) that reacted with a component of the crude capsular antigen died during storage, which made further studies not possible. It was intended to examine the chemical nature of the antigen against which this antibody reacted by crossed-immunoelectrophoresis by heating the capsular antigen to 100^{o}C and treating with periodate and proteases prior to electrophoresis.

The "capsular" antibody had no involvement in the complement mediated killing of bacteria *in vitro*, had no ability to protect mice passively, but could enhance phagocytosis by mouse peritoneal macrophages. Treatment of bacteria with hyaluronidase to decapsulate them did not increase the percentage phagocytosed. In mice, this anti-capsule antibody plays a very small role in their immune mechanisms. This IgM antibody which reacted mainly with the homologous strain in IHA, is more likely to be against a strain-specific surface antigen than the capsular hyaluronic acid.

The hybridoma cells which were producing the anti-77 kDa protein antibody ceased secreting antibody thus limiting more detailed studies of this antibody. From the results it was apparent that

the IgG antibody was not bactericidal and not protective in mice. This is in contrast to the findings of Lu *et al* (1987b) where hyperimmune rabbit serum containing a high titre of IgG antibody directed mainly against the 37.5 kDa protein was protective in rabbits. However, the mechanism by which the protection resulted by these antibodies was not defined by those authors.

Five of the eight mAb in this present study could not be characterized, although they all reacted in whole cell ELISA. None of them reacted in IHA or in LPS Western blots indicating that they were not against the capsule or LPS. The failure of recognition of proteins by Western blotting might be an indication of the loss or denaturation of epitopes during the complex process involved in separation on PAGE and transfer to NC. These antibodies may react with undenatured proteins on intact bacteria. The inability to demonstrate protein specificity by the mAb against *Ehrlichia risticii* has also been reported by Shankarappa and Dutta (1989b).

An encouraging finding in this study is the ability of the IgG_3 anti-LPS mAb to protect mice, which is clearly associated with the complement-dependent antibody-mediated lysis of bacteria. This same antibody seems to enhance phagocytosis of *P. multocida* by the peritoneal macrophages. Similarly, two anti-capsule mAbs to *P. haemolytica* A_1 mediating both these mechanisms have been reported by Penaredondo *et al* (1988).

The epitope recognised by the anti-LPS mAb does not appear to be present in 42% of *P. multocida* and the other Gram-negative organisms tested. Correlation ($P < 0.001$) of whole-cell ELISA titres to the bactericidal capacity of this antibody against

different strains, highlights the use of either of these assays to interpret the immune status of mice. From the previous study (Chapter 5), involvement of a cell surface antigen in this mechanism of immunity was suggested, since the bactericidal capacity of mouse polyclonal serum correlated well ($P < 0.001$) with whole-cell ELISA titres. It is now very clear that the LPS is this cell surface antigen and that the anti-LPS antibodies play a major role in the immune mechanisms of mice.

The structural specificity of this monoclonal was found to be directed against the lipid A moiety of the LPS. As lipid A is the most conserved structural element of LPS, a degree of cross-reactivity of the lipid A antibodies can be expected to exist between different species of bacteria (Johns *et al* 1977). However, more recent work has shown that mAbs to lipid A are restrictive in their specificity rather than cross-reactive (Pollack *et al* 1989), which is in agreement with the present findings.

The structural analysis of LPS of two strains showed similar chemical compositions. However, the sugar compositions of the polysaccharide fraction of the two strains were found to be different, while lipid A moities were constituted of identical fatty acids but in varying molar proportions. These findings are in agreement with the other reports that the lipid A is the most conservative part of the LPS molecule (Brade *et al* 1988; Pollack *et al* 1989).

However, it must be noted that the specificity of this anti-LPS mAb as determined by inhibition of ELISA appeared to be directed towards the lipid A. In this case, it could be anticipated that

the differences may occur in the lipid A moiety of these two strains. Whether the different molar proportions of the 2 fatty acids (C14:0 and C16:0) detected in strain W674 were responsible for this specificity of the mAb is questionable. As pointed out by Brade *et al* (1988), in any serological test the ability of lipid A to generate a completely different physicochemical surrounding may have influenced the results obtained in the ELISA inhibition in this study. They also added that the lipid A could interact with plastic surfaces in the ELISA test which may change the supramolecular structure (conformation) of lipid A.

Although the studies carried out on LPS in this thesis were limited and could not arrive at any conclusion, they opened up avenues for future research, which will lead to better understanding of the immunogenicity and antigenicity of the LPS of *P. multocida*.

It will be interesting to study the structure of the LPS of a greater number of selected strains of *P. multocida* based on the ELISA results obtained in this study. Also, more advanced methods such as nuclear magnetic reasonance (NMR) spectrometry and GC - mass spectrometry techniques would have been helpful to confirm the structural arrangements of the LPS of these strains.

CHAPTER 7

General Discussion

The studies described in this thesis were aimed at identifying the protective antigen(s) and the effector mechanism(s) of immunity to *P. multocida* in mice.

The antigens responsible for homologous and heterologous protection are not yet defined. Attempts have been made to identify the subcellular constituents of *P. multocida* in the hope that better vaccines than those currently available could be developed. Capsular polysaccharides of serotypes B and E are protective against haemorrhagic septicaemia (Penn and Nagy 1974; Nagy and Penn 1976). However, capsular substances may not be important in protection against fowl cholera because immunity can be induced by non-capsulate as well as capsulate avian strains of *P. multocida* (Heddleston *et al* 1964). Tissue vaccines or vaccines containing *in vivo* grown cells induced better immunity than did ordinary bacterins, and were also cross-protective (Heddleston and Rebers 1972; Rebers and Heddleston 1977).

Therefore, investigations aimed at simulating the antigen expression that would probably occur in cells grown *in vivo* and their protective ability in mice were carried out. Iron scarcity is one of the factors bacteria have to overcome in the host. In order to assimilate bound iron from host-binding proteins most bacteria produce siderophores (Barclay 1985) and/or high-molecular mass outer membrane proteins (Overbeeke and Lugtenberg 1980). Certain other bacteria e.g. *Bordetella pertussis* and *Neisseria*

meningitidis seem to obtain iron directly from the host iron-binding proteins via cell surface mechanisms (Redhead *et al* 1987; Schryvers and Morris 1988). These authors have suggested that the iron regulated proteins or receptors on bacteria for lactoferrin or transferrin may be possible vaccine candidates.

Investigations into the expression of these proteins by *P. multocida* under iron limitation *in vitro* and *in vivo* were carried out. *P. multocida* type A of bovine origin recovered from the pleural fluid of experimentally infected lambs expressed two unique outer membrane proteins of molecular masses of 84 and 87 kDa. These proteins were also expressed in cells grown *in vitro* under iron depleted conditions in nutrient broth containing 2,2'-dipyridyl, but not in bacteria grown in nutrient broth only. Ikeda and Hirsh (1988) reported the expression of a protein of 84 kDa by all the somatic types of *P. multocida* when they were grown in BHIB containing 2,2'-dipyridyl. This was in agreement with the findings of the present study. However, in addition to this 84 kDa protein, two other proteins of 80 and 96 kDa were observed by Snipes *et al* (1988) when turkey *P. multocida* was grown in turkey plasma at 41^{o}C and in BHIB with 2,2'-dipyridyl. These two proteins were not observed in this present study.

Mice immunized with heat killed organisms which had been grown in nutrient broth, nutrient broth containing 2,2'-dipyridyl, horse serum and *in vivo* demonstrated good homologous protection. There was a suggestion that the vaccine which contained cells grown in nutrient broth with 2,2'-dipyridyl induced a slightly higher protection against challenge than the others. However, this

protection was not significantly better on statistical analysis. It is apparent that in mice all vaccines give good protection and therefore it was difficult to show better protection. A greater antibody response was also observed in sera of mice immunized with bacteria expressing iron regulated proteins compared to the other growth condition as determined by ELISA. This higher antibody response can not be accounted for by the presence of iron regulated proteins as no antibody response against the 84 and 87 kDa proteins could be demonstrated in Western blots. Instead, growth of *P. multocida* under iron-restricted conditions *in vitro* may have produced a different antigenic profile to which mice responded. This is supported by the appearance of two additional low molecular mass proteins recognized by the sera from this same group of mice.

In the same experiment, when the virulence (LD_{50}) of bacteria grown under different cultural conditions was tested, it was found that nutrient broth grown and *in vivo* cells were the most virulent in mice. If outer membrane proteins are responsible for the virulence, then it could be expected that bacteria expressing iron-regulated proteins would be more virulent. This is the case with certain *Yersinia* species, where presence of iron-regulated proteins (IRP) correlated with the degree of virulence of the species (Carniel *et al* 1987). In the present study the presence of IRP did not correlate with the virulence of bacteria. Since the capsule of *P. multocida* is also not related to virulence (Curtis *et al* 1980) it may be the LPS of the organism that is associated with virulence as indicated by Poxton and Arbuthnott (1990). More work has to be done on the expression of IRP by *P. multocida* and their role in immunity with special reference to cattle.

Studies which were carried out with a view to identifying the effector mechanism(s) of immunity to *P. multocida* indicated that the complement-mediated bactericidal activity of immune mouse serum is an important mechanism. In this study the protection in mice was clearly associated with the bactericidal antibodies. The correlation of bactericidal capacity with whole-cell ELISA suggested that cell surface antigens were involved in stimulating bactericidal antibodies. In most other Gram-negative bacteria LPS has been identified as this target antigen (Taylor 1983; Sutherland 1988).

Although immune mouse serum showed the ability to opsonophagocytose *P. multocida*, it was to a very low percentage in comparison to what was observed for *P. haemolytica* (Sutherland, thesis in preparation 1989). Therefore, it is not clear whether the phagocytosis enhanced by immune mouse serum is significant in protection.

The anti-LPS mAb gave substantial evidence that LPS has a crucial role in protection of mice against *P. multocida* infection. The association observed between the whole-cell ELISA titres, bactericidal capacity and Western blotting of LPS of different isolates of *P. multocida* suggested that the complement-mediated antibody-dependent killing is an important mechanism of immunity to *P. multocida* in mice. This also indicated that LPS is the target antigen in this mechanism of immunity.

LPS has been implicated as the important immunogen when complexed with protein in chickens, mice and rabbits (Heddleston *et al* 1972; Rebers *et al* 1980; Rebers and Heddleston 1974; Ganfield

et al 1976; Syuto and Matsumoto 1982; Tsuji and Matsumoto 1988 (a) and (b)). Purified LPS was toxic and was found to be immunogenic only in chickens and not in mice or rabbits (Rebers *et al* 1980). The requirement of LPS in ribosomal vaccines for the induction of immunity was demonstrated by Phillips and Rimler (1984). All these studies indicate the importance of LPS in inducing immunity against *P. multocida*. Although very little work has been carried out in cattle on this aspect, a report of detection of elevated levels of antibody to a crude LPS preparation and certain proteins in sera of cattle vaccinated against haemorrhagic septicaemia also suggested the role of LPS in immunity in *P. multocida* (Johnson *et al* 1989). Whether the presence of these antibodies correlate with protection has not been studied by these authors.

The passive protection experiments carried out in mice also indicated that only anti-LPS antibodies could protect mice while the anti-capsule and the anti-protein (77 kDa) were not protective against the challenge. Although the IgG_3 anti-LPS mAb enhanced phagocytosis by peritoneal macrophages, it is evident that the mechanism by which it brings about protection is complement-mediated bacteriolysis. Its structural specificity towards lipid A, as suggested by ELISA inhibition, supports the assumption that the fixing of complement close to the cell membrane causes lysis of bacteria. This hypothesis is furher-supported by the findings of an anti-LPS mAb raised against *P. haemolytica* reactive against the more distal polysaccharide moiety being not bactericidal (Wilson *et al* 1989, personal communication).

The structural studies of LPS and their lipid A and polysaccharide fractions from two strains of *P. multocida* was aimed at expanding the knowledge on the structure of LPS in relation to the specificity of the mAb. From the techniques employed, although differences between the two strains were detected especially in the polysaccharide fraction, no firm conclusions could be made. The lipid A moieties of the two strains contained the same fatty acids but in different molar proportions. Hydroxy fatty acids, which are typical of LPS of other bacteria, were also present.

Future research therefore should concentrate on a more detailed study of the structure of LPS from more strains: which react with the anti-LPS mAb and those which did not react, using more powerful techniques. The protective ability of either crude or purified LPS must be studied in cattle and the effector mechanisms of immunity also need more extensive investigations. Since LPS is the criterion for somatic serotyping of *P. multocida* more attention has to be placed on this complex molecule prior to planning any future animal experiments. As it is well known that the capsular type A strains consist of several different somatic (O) types, a wide range of these (O) types must also be investigated in detail. This will be of value in the rational development of cattle vaccines.

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Publications arising from thesis

1. Bactericidal activity in the sera of mice vaccinated against *P. multocida*

2. Production and characterization of monoclonal antibodies against *P. multocida* type A.

3. Immunological properties of a monoclonal antibody specific for lipopolysaccharide of *P. multocida* type A.

APPENDIX 1

1.1. Checker-board titration of NB grown *P. multocida* (10^8 cfu/ml) as antigen and mouse immune serum from chamber implanted mice as the standard positive serum

OD_{492} for each dilution

Antigen dilution	Serum dilution 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
10^{-1}	0.96	0.92	0.87	0.72	0.81	0.67
10^{-2}	0.79	0.61	0.49	0.49	0.51	0.39
10^{-3}	0.88	0.61	0.24	0.30	0.39	0.19
10^{-4}	0.52	0.43	0.26	0.24	0.20	0.11
10^{-5}	0.31	0.27	0.17	0.19	0.13	0.07
10^{-6}	0.19	0.14	0.09	0.08	0.04	0.00

Antigen dilution 10^{-1} (10^7 cfu/ml) and

Antibody dilution 10^{-2} were selected

1.2. Screening of hybrids for antibody production by indirect ELISA.

Optical densities of twenty positive hybrids

Cell line	OD 492nm	Cell line	OD 492 nm
1/1	1.157	1/11	0.531
1/2	0.586	1/12	0.571
1/3	0.622	1/13	0.807
1/4	1.847	1/14	0.560
1/5	0.511	1/15	0.577
1/6	1.206	1/16	1.084
1/7	1.413	1/17	0.536
1/8	0.547	1/18	0.518
1/9	0.802	1/19	0.523
1/10	0.917	1/20	0.594

Standard positive polyclonal serum when doubly-diluted from 1 in 100 gave:- 1.925, 1.915, 1.842, 1.377, 0.758, 0.406, 0.136, 0.058

1.3. ELISA optical densities of clonings tested.

No. clonings tested	OD_{492} with cell lines: 1/2	1/4	1/8	1/13	1/15	1/18	1/19	1/20
1	0.009	*0.244	0.059	0.131	0.180	0.331	0.071	0.155
2	0.220	0.226	0.095	0.194	0.147	0.336	0.032	0.142
3	0.041	0.210	0.064	0.199	0.085	0.348	0.276	0.293
4	0.200	0.000	0.094	0.176	0.179	0.315	0.145	0.324
5	0.002	0.000	0.038	0.218	0.159	*0.328	0.141	0.239
6	0.218	*0.213	0.023	*0.296	0.101	0.344	0.034	0.016
7	0.229	0.201	0.035	*0.274	0.113	0.203	0.043	0.313
8	0.063	0.119	0.053	0.080	0.023	0.282	0.033	0.262
9	0.021	0.015	0.078	0.050	*0.277	0.357	0.049	0.142
10	0.214	0.118	0.144	0.174	*0.218	0.279	0.005	*0.336
11	0.018	0.227	0.101	0.102		0.305	*0.211	0.261
12	0.192	0.018	0.092	0.104		0.305	*0.137	0.362
13	0.214		0.054	0.081		*0.402	0.092	0.319
14	0.412		0.101			0.325	0.040	0.184
15	*2.291		*0.246			0.313		0.048
16	*0.497		*0.110			0.368		*0.403
17			0.111			0.463		0.127
18						0.230		0.084
19								0.018

Standard positive serum from chamber implanted mice titrated in doubling dilutions from 1 in 100 gave OD_{492} of 0.785, 0.686, 0.578, 0.380, 0.328, 0.296, 0.294, 0.243

* Indicates the clones that were selected for further study

APPENDIX 2

2.1. List of bacterial strains tested against the anti-LPS mAb by ELISA

P. multocida	X12	X1113	A513
	W669	A341	A514
	A33	X223	Type A (10322)
	X120	A757	Type B (10323)
	A26	Q110	Type D (10325)
	X9	W735A	Type E (10326)
	W674	A34	*K. oxytoca* (A405/2)
	W829	A515	*S. montevideo*(A459/1)
	X110	W666	*S. arizonae* (A459/2)
	X200	X1016	*S. derby* (A459/27)
	A87	A872	*Ser.liquefaciens* (A405/1)
	X1122	812	*E. coli* (A405/3)
	X1053	396	*Y.pseudotuberculosis* (A1218)
	A55	A586	*P. haemolytica* A1
	A394	W598	*P. haemolytica* A2
	X188	W861	*P. haemolytica* T3
	A36	X1057	*P. haemolytica* T4
	A440	X142	*P. haemolytica* A5
	A419	A82	*P. haemolytica* A6
	A1174	A79	*P. haemolytica* A7
	A848	W828	*P. haemolytica* A8
	X109	X1056	*P. haemolytica* A9
			P. haemolytica T10
			P. haemolytica A11
			P. haemolytica A12
			P. haemolytica A13
			P. haemolytica A14
			P. haemolytica T15
			P. haemolytica A16
			L. monocytogenes (4B)